UNIVERSITY of OTAGO

Te Whare Wānanga o Otāgo

NEW ZEALAND

Measuring Markers of Immune Response in Patients Treated with Nivolumab (Opdivo®) and Pembrolizumab (Keytruda®)

Author:
Leah BUTT
(ID: 5660278)

Supervisors:
Dr. Margaret CURRIE
Dr. Barry HOCK
Dr. Matthew STROTHER

A thesis submitted for the degree of
Bachelor of Biomedical Science with Honours
University of Otago, Christchurch

2017

Abstract

Immune checkpoint inhibitors (ICIs) have drastically improved the clinical outcome for many cancer patients. However, not all patients treated with ICIs show a clinical response and the development of a unique spectrum of adverse events, termed immune-related adverse events (irAEs), restricts their oncological applicability. Additionally, the production of anti-drug antibodies (ADAs) may negatively influence patient outcome, but their role during therapy is yet to be established due to limitations in standard detection techniques. The primary aim of this exploratory study was to identify biomarkers that predict patient outcome to prevent a proportion of individuals exposed to a potentially ineffective and/or harmful therapy. It was hypothesized that patients who develop anti-drug antibodies experience a decrease in treatment efficacy and/or an increase in toxicity. Furthermore, patients with differing outcomes in terms of response, survival and the development of toxicity may display distinct clinicopathological characteristics.

A retrospective review was performed on 32 patients undergoing nivolumab or pembrolizumab monotherapy for metastatic melanoma. Blood serum trough samples from 8 pembrolizumab-treated patients were analysed using in-house developed ELISA's to measure pembrolizumab and anti-pembrolizumab antibody levels. Of the patients reviewed, 23 (72%) were ineligible for inclusion in initial clinical trials of ICI drugs. 29 patients (91%) experienced irAEs and 13 (41%) progressed during treatment. No clinicopathological variables were found to significantly predict patient outcome. Anti-pembrolizumab antibodies were detected in one patient and correlated with decreased blood serum drug levels. In this patient case, the individual responded to treatment according to RECIST, but, developed irAEs (pneumonitis and infusion reactions).

This study indicates that, patients who are ineligible for initial clinical trials may effectively be treated with immune checkpoint inhibitors. Further investigation in a larger cohort is required to determine the prevalence and role of anti-ICI antibodies during ICI-therapy.

Acknowledgements

This thesis would not have been possible without a number of people and I would like to take the time to thank all those who have helped out in one way or another. Firstly, a huge thank you to my primary supervisor, Dr. Margaret Currie, for all her help, feedback and encouragement throughout this year. You have been great. Thank you very much for the opportunity to work on this project, guiding me through it and introducing to me to the world of research.

A big thank you to my co-supervisor Dr. Barry Hock, who has been incredibly helpful throughout this project and provided fundamental preliminary work for this thesis. Thank you for sharing your expertise, time and statistical knowledge. Thanks also to the rest of the Haematology Research group for their role in work prior to this project and a special thanks to Liping for helping me find my way around the lab.

I would also like to express my thanks to my co-supervisor, Dr. Matthew Strother, for taking the time to answer any questions and helping me understand the clinical aspects of this project. Thank you also to the all of the clinical team involved in recruiting patients and collecting blood samples for this project.

Along with my supervisors, I would also like to acknowledge the rest of the Mackenzie Cancer Research group. I have very much enjoyed being part of this group. Thank you all for being so welcoming, lending a helping hand whenever I needed it and supplying an endless amount of cake!

Thanks as well to all the people behind the scenes of the BBiomedSci course. Thank you to Prof Madhav Bhatia, Prof Mark Hampton, Prof Martin Kennedy, Alice Milnes and Linda Kerr for all the organisational and administrative work you do to keep the course running smoothly throughout the year. I would also like to thank the members of the Department of Pathology who kindly contributed their time to teaching at the beginning of the year.

Finally, a special thanks to all my family and friends for their unwavering support, not only this year, but in years before and in years to come. I cannot thank you all enough.

Contents

List of figures

List of tables

List of abbreviations

Abbreviation	*Description*
ACE	affinity capture elution
ADA	anti-drug antibody
APC	antigen-presenting cell
BMI	body mass index
BSA	bovine serum albumin
CBC	complete blood count
CEACAM1	carcinoembryonic antigen related cell adhesion molecule 1
CT	computerized tomography
CTCAE	common terminology criteria for adverse events
CTLA-4	cytotoxic T-lymphocyte antigen 4
E. coli	Escherichia coli
ELISA	enzyme-linked immunosorbent assay
FDA	food and drug administration
GAH	goat anti-human
GI	gastrointestinal
HRG	haematology research group
HRP	horseradish peroxidase
HSMA	homogeneous shift mobility assay
ICI	immune checkpoint inhibitor
IgG	immunoglobulin
IL-17	interleukin 17
irAE	immune-related adverse event
iRECIST	immune-related response evaluation criteria in solid tumours
irRC	immune-related response criteria
kg	kilogram
KIR	killer-immunoglobulin-like receptor
LMR	lymphocyte to monocyte ratio
mAb	monoclonal antibody
MEK	mitogen-activated protein kinase kinase
mg	milligram
MHC	major histocompatibility complex

mL	millilitre
MP	milk powder
n	sample size
NLR	neutrophil to lymphocyte ratio
nm	nanometer
No.	number
NSCLC	non-small cell lung cancer
o/n	overnight
PBS	phosphate-buffered saline
PD-1	programmed cell death protein 1
PD-L1	programmed death-ligand 1
PD-L2	programmed death-ligand 2
PFS	progression-free survival
Q2W	every 2 weeks
Q3W	every 3 weeks
RECIST	response evaluation criteria in solid tumours
SA	streptavidin
SE-HPLC	size exclusion high pressure liquid chromatography
™	Trademark
TMB	3,3',5,5' tetramethylbenzidine
TNFα	tumour necrosis factor alpha
µg	microgram
µL	microliter
%	percentage
℃	degree Celsius
®	registered trademark

Chapter 1

1 Introduction

Immunotherapy is rapidly cementing its position as the fourth pillar of cancer treatment.[1] In contrast to the three traditional cancer treatments (surgery, radiation and chemotherapy) which target cancer cells, the mechanistic target of immunotherapy is the patient's natural immune cells.[2] One of the first examples of immunotherapy dates back to 1891, when Dr. William Coley successfully treated sarcoma patients by injecting bacterial toxins to evoke an immune response.[3,4] However, multiple deaths and the concurrent arrival of radiation and chemotherapy halted the momentum of immunotherapy.[5] Nonetheless, continued efforts investigating a number of immunotherapeutic approaches ensued over the following century. The result, a myriad of potential immunotherapeutic treatments ranging from cytokines and small molecules to vaccines and engineered immune cells, many of which have yielded modest results and limited clinical applicability.[6,7,8] That is, until the recent success of a novel class of immunotherapeutic agents known as immune checkpoint inhibitors (ICIs). The development and introduction of ICIs has drastically improved the clinical outcome for many cancer patients, particularly melanoma patients.[5,9,10,11,12]

New Zealand has the highest rate of melanoma in the world and skin cancers (melanoma plus non-melanoma cancers) are the most common cancers in New Zealand, accounting for 80% of all new cancer diagnoses each year. Although melanoma diagnoses occur less frequently than non-melanoma skin cancer diagnoses, they represent a bleaker outcome for patients, making up 70% of all skin cancer deaths.[149] The approval and transition of ICIs into clinical practice is expected to improve survival rates of New Zealanders diagnosed with melanoma.[150] However, not all patients treated with ICIs show clinical response, and ICIs generate a unique spectrum of side effects that differ from that of traditional cancer treatments.[10,11] These effects, termed immune related adverse events (irAEs), resemble autoimmune disease and target various tissues (e.g skin, gastrointestinal tract, endocrine organs) to produce a multitude of manifestations (e.g rash, colitis, endocrine dysfunction).[13,14,15,16,17] Moreover, irAEs can be debilitating and lead to the discontinuation of therapy, irreversible organ damage and/or death.[11,18] Interestingly, however, the appearance of irAEs has been associated with an improved clinical outcome to ICI-therapy.[100,103,104,105]

The first approved checkpoint inhibitor, ipilimumab, targets cytotoxic T-lymphocyte antigen 4 (CTLA-4), producing durable responses in patients with advanced melanoma. Disadvantageously, however, approximately one third of patients treated with Ipilimumab experience severe or life-threatening effects, referred to as grade 3 or 4 events, respectively.[2,10] Inhibition of an alternative

immune checkpoint, programmed death 1 (PD-1), has produced a more favourable benefit-to-risk ratio, leading to the FDA-approval of two anti-PD-1 antibodies, Nivolumab and Pembrolizumab.[11,19,20,21] However, as with all therapeutic antibodies, unwanted immunogenicity and more precisely, the production of anti-drug antibodies (ADAs) towards ICIs may alter this benefit-to-risk ratio, limiting their success.[147]

The overall goal of this project was to improve our understanding of patient immune response to ICIs and optimise their clinical use. The first aim of this study was to investigate clinicopathological indicators of patient outcome through the retrospective review of patients undergoing anti-PD-1 therapy. The second aim was to use an in-house developed ELISA to measure drug and anti-drug antibody levels in patients undergoing ICI-therapy and, correlate the resulting laboratory data with clinicopathological characteristics collected for each patient (including age, gender and patient outcome). Thirdly, we aimed to identify serum markers that could be used to predict patient outcome, particularly with regards to the development of irAEs. Currently there are no known biomarkers predictive of toxicity in patients treated with PD-1 inhibitors. By identifying predictive biomarkers of toxicity, the underlying mechanisms and therefore causes of irAEs may be determined. Moreover, such biomarkers would allow early detection and improved management of these reactions, as well as the avoidance of unnecessary toxicity and healthcare costs. Therefore, to prevent a proportion of patients exposed to an ineffective and potentially harmful therapy, this project focuses on identifying biomarkers of patient outcome. Of particular interest, were biomarkers of patient response, survival and development of toxicity in patients with metastatic melanoma treated with nivolumab or pembrolizumab.

1.1 Cancer and the immune system

Although initially controversial, the major role of the immune system in tumour surveillance is now widely recognised. ‘Cancer immunoediting’, a leading concept describing the immune system’s influence on tumour progression, proposes three distinct phases of immune surveillance: elimination, equilibrium and escape.[22,23,24] Each phase represents a dynamic interaction in which multiple effector cells and secreted cytokines play a pivotal role.[23,25] During the elimination phase, transformed tumour cells are successfully destroyed by the host’s immune system. If unsuccessful, the equilibrium phase can proceed. The host’s immune system is unable to fully eliminate the tumour cells but can adequately restrain their growth, leading to a period of dormancy. Accompanied by appropriate genetic changes these tumour cells can evade the immune system, thereby escaping immunosurveillance. Tumour cells enter the escape phase where they begin to proliferate, progressively forming a clinically detectable tumour.[23,24] Hanahan and Weinburg introduced the ‘Hallmarks of Cancer’ to describe traits that normal cells acquire during their

transformation into cancerous cells and progression into this escape phase. Included in these traits is the ability of a cancer cell to evade immunosurveillance.[26] Multiple ways in which cancer cells achieve this have been theorized.[27] One established mechanism involves the up-regulation of immune checkpoint molecules on cancer cells that promote suppression of the hosts immune system.[28,29] Understanding these mechanisms has allowed the development of therapeutic approaches such as immune checkpoint inhibitors that promote return to the equilibrium or elimination phase, thereby restraining or destroying tumour growth.

1.2 Immune checkpoints

Under normal physiological conditions, immune checkpoint molecules regulate T cell activation to generate an effective immune response, without inducing excessive inflammation or autoimmunity.[30] According to the two-signal model of T cell activation, interactions between co-signaling molecules on the surface of T cells and antigen-presenting cells (APCs) are required for optimal activation of a naive T cell.[31,32] Furthermore, this interaction is believed to determine the amplitude of the T cell response. It is now understood that co-signaling molecules can act through co-stimulatory pathways promoting T cell activation, or oppositely, through co-inhibitory pathways that inhibit T cell responses.[33] Evidence for an inhibitory role of immune checkpoint molecules to maintain immune homeostasis has come from a series of *in vitro* and *in vivo* experiments. In the case of two clinically relevant immune checkpoints, CTLA-4 and PD-1, *in vitro* experiments have demonstrated blocking with antibodies augments T cell responses, suggesting this blockade prevents transmission of an inhibitory signal.[34,35] Additionally, blockade in mouse models of autoimmunity exacerbates autoimmune manifestations supporting a role of these molecules in self-tolerance.[36,37] CTLA-4 knockout mice develop a lymphoproliferative disorder which leads to fatal multi-organ tissue destruction and early death.[38,39] Similarly, mice deficient in PD-1 display multiple autoimmune-like conditions including lupus-like arthritis, glomerulonephritis and encephalomyelitis.[40,41,42,43] Interesting, however, it appears mice do not display patterns of toxicity similar to those of patients undergoing ICI-therapy. Common irAEs observed in ICI-treated patients are rarely seen in CTLA-4 or PD-1 deficient mice, which may explain why initial preclinical animal studies failed to predict these irAEs.[134] Other compelling data has linked defects or functional mutations in CTLA-4 or PD-1 molecules with various autoimmune and inflammatory conditions.[44,45] It is yet to be discovered whether these mutations play a role in the development of toxicity in patients undergoing immune checkpoint inhibitor therapy.

1.2.1 CTLA-4 and PD-1/PD-L1 pathway

The most extensively studied immune checkpoint pathways, the CTLA-4 and PD-1 pathways, have proven to be clinically relevant in cancer therapy.[10,11] Both CTLA-4 and PD-1 belong to the B7 receptor family and are expressed on the surface of T cells.[30] Differentially, however, CTLA-4 and PD-1 interact with different targets inducing distinct signaling pathways.[46,47] CTLA-4, a CD28 homolog, competitively binds B7 on APCs with high affinity.[48] Following initial signaling and activation of a T cell through its T cell receptor, CTLA-4 is up-regulated. Binding of B7 to CTLA-4 rather than CD28 prevents transference of the second co-stimulatory signal required for full activation of T cells. The proportion of CD28:B7 binding and CD28:CTLA-4 binding ultimately determines whether a T cell will become fully activated or undergo anergy. Therefore, an appropriate immune response can be generated without over-activation and autoimmunity or inflammation resulting. Blockade of CTLA-4 prevents binding to B7, shifting the proportion of binding to CD28:B7.[47] This blockade allows greater activation of T cells, and consequently a more intense immune response to be generated. Conversely, PD-1 has been shown to bind either PD-L1 (B7-H1, CD274) or PD-L2 (B7-DC, CD273).[49,50] Within an unhindered immune system, the intended ligand of PD-1 is PD-L1 or PD-L2 expressed on the host's immune cells.[51] Similar to the CTLA-4 pathway, this interaction has been shown to restrict T cell activation preventing over-activation and maintaining self-tolerance.[47] However, a series of experiments have confirmed PD-1 can alternatively bind PD-L1 or PD-L2 expressed on tumour cells.[52,53,54,55] Furthermore, evidence suggests expression of PD-L1 or PD-L2 is a mechanism by which cancer cells exploit the PD-1 immune checkpoint pathway to evade the immune system. Through binding and inhibition of PD-1 by PD-L1 or PD-L2 expression, cancer cells are able to generate an immunosuppressive effect. As a result, cancer cells are able to evade the immune system, allowing persistence and progression within the host.[56,57]

Due to the differences described above, it is thought that immune checkpoint molecules function at different stages of the immune response. In contrast to CTLA-4, which is thought to regulate T cells at an early initial activation stage, PD-1 is believed to regulate the immune response at multiple stages, particularly at a later stage regulating effector T cells in peripheral tissues.[47] Figure 1.1 depicts the stages of the cancer-immunity cycle at which therapeutic agents, including CTLA-4 and PD-1 inhibitors are believed to exert their effects. The different stage at which CTLA-4 inhibitors act compared to PD-1/PD-L1 inhibitors is believed to contribute to differences in drug efficacy and toxicity.[58]

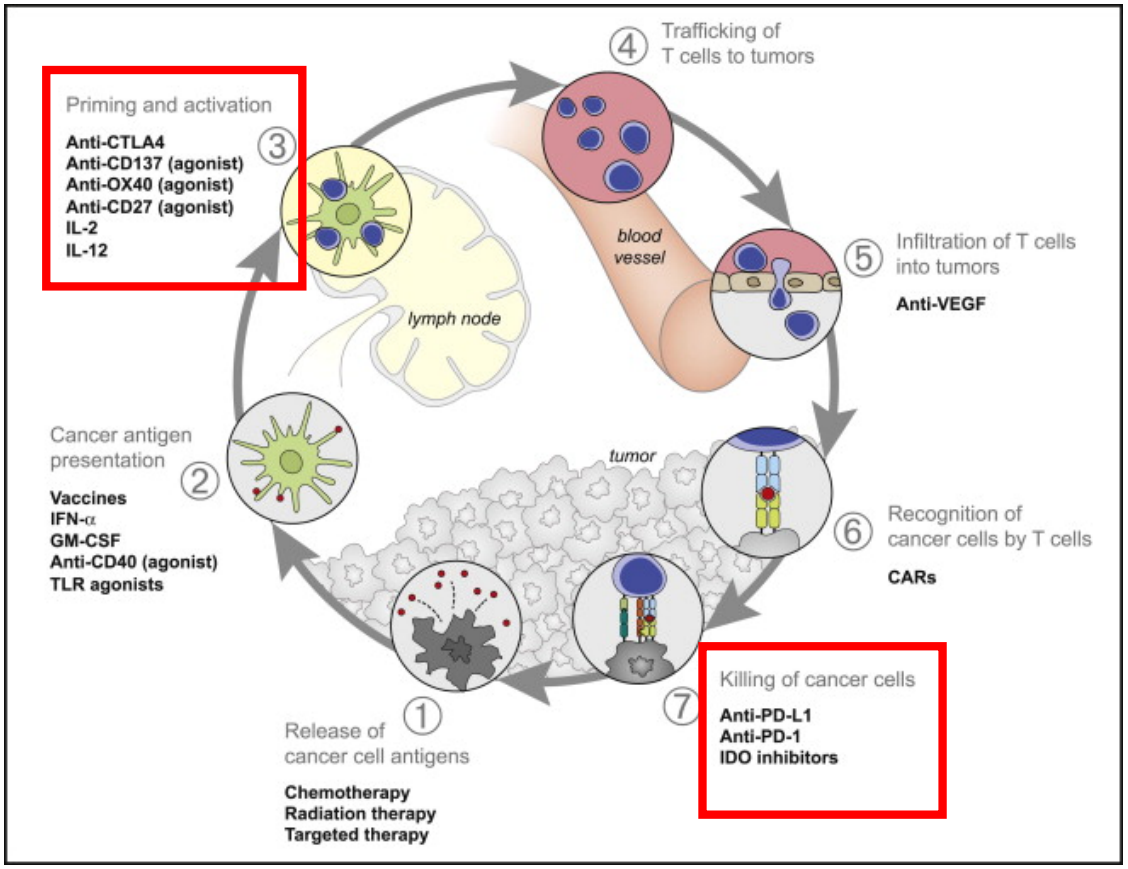

Figure 1.1: Stages at which therapies might affect the cancer-immunity cycle. Anti-CTLA4 and anti-PD-1/PD-L1 antibodies are proposed to affect the cancer-immunity cycle at stage 3 and 7, respectively. Adapted from Chen and Mellman (2013).[58]

1.3 Immune checkpoint inhibitors

The recognition that immune checkpoint molecules can be blocked by antibodies to reverse immune suppression and therefore generate anti-tumour effects has prompted their emergence as therapeutic targets in cancer treatment.[59,60] Early research has elucidated the chemical structures, signaling pathways and interactions of multiple immune checkpoint molecules within the immune system and tumour microenvironment.[61,62,63] As a result, six immune checkpoint inhibitors have been developed and FDA-approved for the treatment of several cancer types.[5,64,65] The two best characterised checkpoint pathways, the CTLA-4 and PD-1/PD-L1 pathway, are the main targets of clinically available inhibitory antibodies, as shown in Table 1.1, which summarises FDA-approved immune checkpoint inhibitors.[5] Although, in an area of active research, promising data is emerging showing currently approved antibodies and newly developed antibodies targeting alternative checkpoints can exert anti-tumour effects in other tumour types.[66,67,68,69,70] For example, data has shown Nivolumab exhibits promising activity in hepatocellular carcinoma and acute myeloid leukemia.[66,67] Additionally, Lirilumab, a monoclonal antibody that targets KIR immune checkpoints, has demonstrated activity in head and neck cancer.[69]

Table 1.1: FDA-approved immune checkpoint inhibitors. Adapted from Sweis and Luke (2017), with information provided by the U.S Food & Drug Administration.[5,64,65]

Target	Drug	Approval year	Indication
CTLA-4	Ipilimumab	2011	Melanoma (metastatic/unresectable)
		2015	Melanoma (adjuvant)
PD-1	Pembrolizumab	2014	Melanoma (metastatic/unresectable)
		2015	Non-small cell lung cancer
		2016	Head and neck cancer
	Nivolumab	2014	Melanoma (metastatic/unresectable)
		2015	Non-small cell lung cancer
		2015	Renal cell carcinoma
		2016	Hodgkin Lymphoma
		2016	Head and neck cancer
		2017	Urothelial cancer
PD-L1	Atezolizumab	2016	Urothelial cancer, Lung cancer
		2016	Non-small cell lung cancer
	Avelumab	2017	Merkel cell carcinoma
		2017	Urothelial cancer
	Durvalumab	2017	Urothelial cancer

1.3.1 Monotherapy

Ipilimumab (Yervoy®, Bristol Myers-Squibb), the first and currently only approved checkpoint inhibitor targeting CTLA-4, is a humanized immunoglobulin G1 monoclonal antibody.[71] Clinical trials in various tumour types have demonstrated Ipilimumab is effective in melanoma patients, but fails to produce clinical efficacy in other cancers.[10,72,73,74] In contrast, successively approved inhibitors, Nivolumab (Opdivo®, Bristol Myers-Squibb) and Pembrolizumab (Keytruda®, Merck) are humanised IgG4 monoclonal antibodies, highly selective for human PD-1.[75,76] Unfortunately, although a number of patients experience favourable outcomes, many patients don't respond to treatment with ICIs.[10,11] For example, in a phase III trial involving ipilimumab-treated melanoma patients, only 1.5% of patients achieved a complete response and a further 9.5% achieved a partial response. Disadvantageously, 59.3% of patients experienced progressive disease.[10] Therefore, a huge demand exists for markers that will include (or exclude) patients from treatment based on their likelihood to respond to treatment or risk of developing toxicity.

1.3.2 Combination therapy

Considering CTLA-4 and PD-1 act through distinct signaling pathways, it was theorised that inhibition of both CTLA-4 and PD-1 could produce enhanced anti-tumour responses.[47] For that reason, multiple trials examining the effectiveness of combination therapies involving ICIs are in

progress. Additionally, it is unknown whether the application of ICIs with traditional cancer treatments may provide a benefit to patients. Thus, these trials are not only investigating ICIs in combination with one another, but also with conventional cancer treatments including chemotherapy and radiation.[77] Following a randomised, double-blind, phase III study, the first combination treatment of Ipilimumab plus Nivolumab was FDA-approved for the treatment of advanced melanoma in 2015. In patients with advanced melanoma treated with either Nivolumab alone or in combination with Ipilimumab, a greater response rate was found in the combination treatment group. Unfavourably, however, the incidence of grade 3-4 irAEs was significantly higher in the combination treatment group compared to monotherapy with either agent.[78] To date, all irAEs observed with combination therapy have been observed in monotherapy suggesting homogeneous underlying mechanisms.

1.4 Therapeutic antibodies

The clinical applicability of antibodies spans far wider than their use as anti-cancer agents. Preceding their development as immunotherapeutic checkpoint inhibitors, antibodies have been engineered to treat a wide range of indications.[136,141,144] Favourable properties including long serum half-lives and the ability to bind diverse targets with high specificity and affinity have garnered attraction for drug development.[136] To date, all monoclonal antibodies approved for clinical use are of the IgG isotype of immunoglobulins (Figure 1.2).[136,137] This classification is based on their similar basic structure, in which each antibody consists of four polypeptide chains (two heavy chains and two light chains) held together by disulfide bonds to form a large heterodimeric protein (~150kDa). Each light chain consists of a variable domain (V_L) and a constant domain (C_L). Similarly, each heavy chain consists of a variable domain (V_H) but also three constant domains (C_{H1}, C_{H2} and C_{H3}). The Fab (fragment antigen binding) fragment, which allows the antibody to bind to the antigen, contains the complete light chains, as well as the V_H and C_{H1} heavy chain domains. The remaining C_{H2} and C_{H3} domains form the Fc fragment (fragment crystallizable), which engages with cell surface receptors.[137] As mentioned previously, nivolumab (146 kDa) and pembrolizumab (149 kDa) are further classified into the IgG4 subclass based on differences in sequence, structure and effector functions from the three alternative subclasses; IgG1, IgG2 and IgG3.[75,76,137] Additional modifications to their structure, made possible through advances in antibody engineering, have permitted the humanization of these antibodies. Generation of therapeutic antibodies is routinely achieved from xenogenic sources and therefore, their structure contains sequences that may elicit an immune response in humans. Humanization is a well-established technique that modifies non-human sequences to increase their similarity to naturally-occurring human antibodies, ultimately reducing unwanted immunogenicity of mAbs when

administered to humans.[136] Through the incorporation of suitable manipulations with basic structural properties, immune checkpoint inhibitors function as effective therapeutic agents.

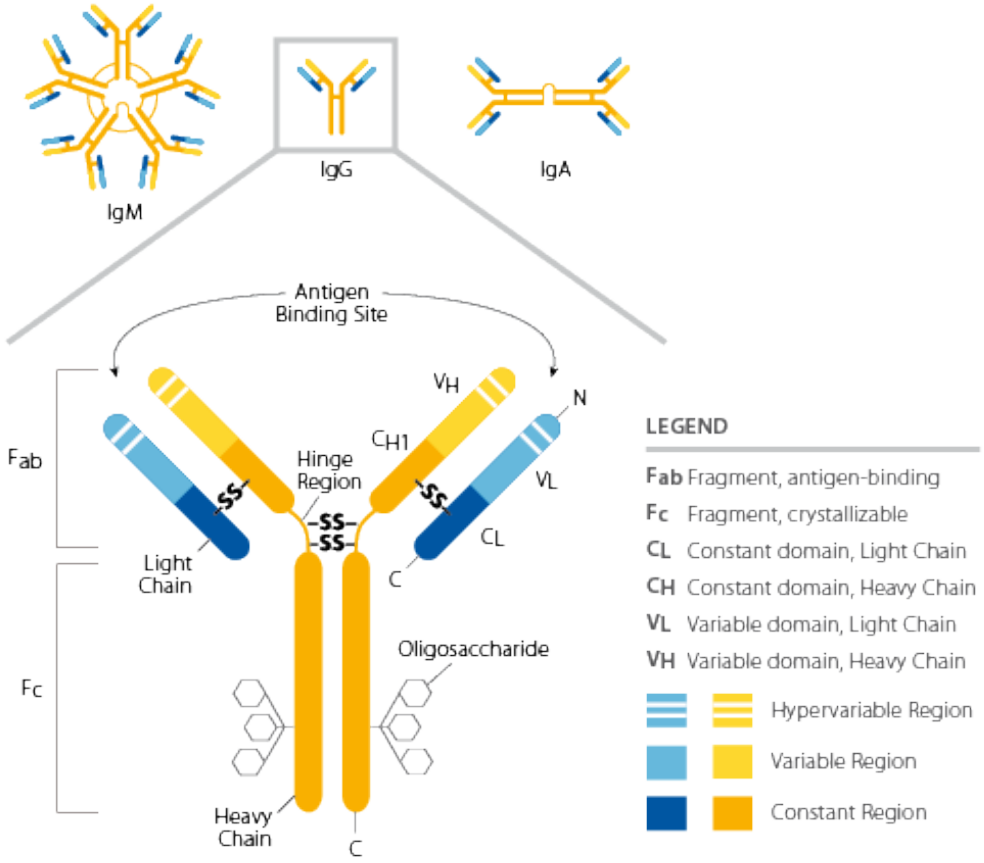

Figure 1.2: Generalized structure of an immunoglobulin (IgG). Reprinted from ThermoFisher Scientific. [138]

1.4.1 Anti-drug antibodies

Nivolumab and pembrolizumab were engineered as humanized monoclonal antibodies (mAbs), to reduce the risk of developing anti-drug antibodies (ADAs).[75,76,137] However, particularly with repeated infusions, administration of mAbs to patients may trigger undesirable immunogenicity, leading to ADA formation.[136, 137] The subsequent binding of ADAs to mAbs has been linked to a decrease in drug efficacy and/or an increase in toxicity events for a wide range of therapeutic antibodies.[140, 142, 144, 152]

In a study conducted by Vultaggio et al., an association between the formation of antibodies towards infliximab, a therapeutic antibody used to treat autoimmune diseases, and an increased risk of hypersensitivity reactions was reported.[140] A number of further studies have reported a loss of efficacy in the presence of ADAs, including a study involving the first approved fully humanized mAb, adalimumab. In this study, anti-adalimumab antibodies were detected in 17% of patients and of these patients, 80% were non-responders.[145] The change in response observed in these studies is proposed to originate from the formation of ADA-mAb immune complexes. Immune complexes

reduce efficacy of treatment by increasing clearance and reducing the bioavailability of the therapeutic antibody. Additionally, immune complexes can promote toxicity through various mechanisms including activation of the complement cascade, Fc receptors, and various inflammatory pathways.[158] Figure 1.3 summarizes the central role of immune complexes within the immune system and capacity to exacerbate an ADA response. Furthermore, a subset of ADAs referred to as neutralizing antibodies, bind to epitopes situated directly within or near the active site of the therapeutic antibody altering the interaction with its target.[137] With regards to anti-PD-1 inhibition, the binding of nivolumab or pembrolizumb to PD-1 may be compromised, diminishing their blocking effect and ultimately their therapeutic effect.

To maximize the anti-tumour effect of ICIs, an optimum concentration of drug must be available to the patient. Patients who generate ADAs towards ICIs may be at an increased risk of non-responding to treatment or developing adverse effects. In patients undergoing ICI-therapy, it is yet to be determined whether anti-drug antibodies induce these altered responses and, if so, to what extent. Monitoring of therapeutic antibodies and their respective anti-drug antibodies has become common practice for inflammatory bowel disease and arthritis patients (adalimumab and infliximab-treated patients), and is used to guide treatment regimens.[151] However, testing of these parameters is not routinely performed for patients receiving immune checkpoint inhibitors. Accurate monitoring of both serum ICI levels and ADA levels will determine at-risk patients, improving usage of immune checkpoint inhibitors in a clinical setting.

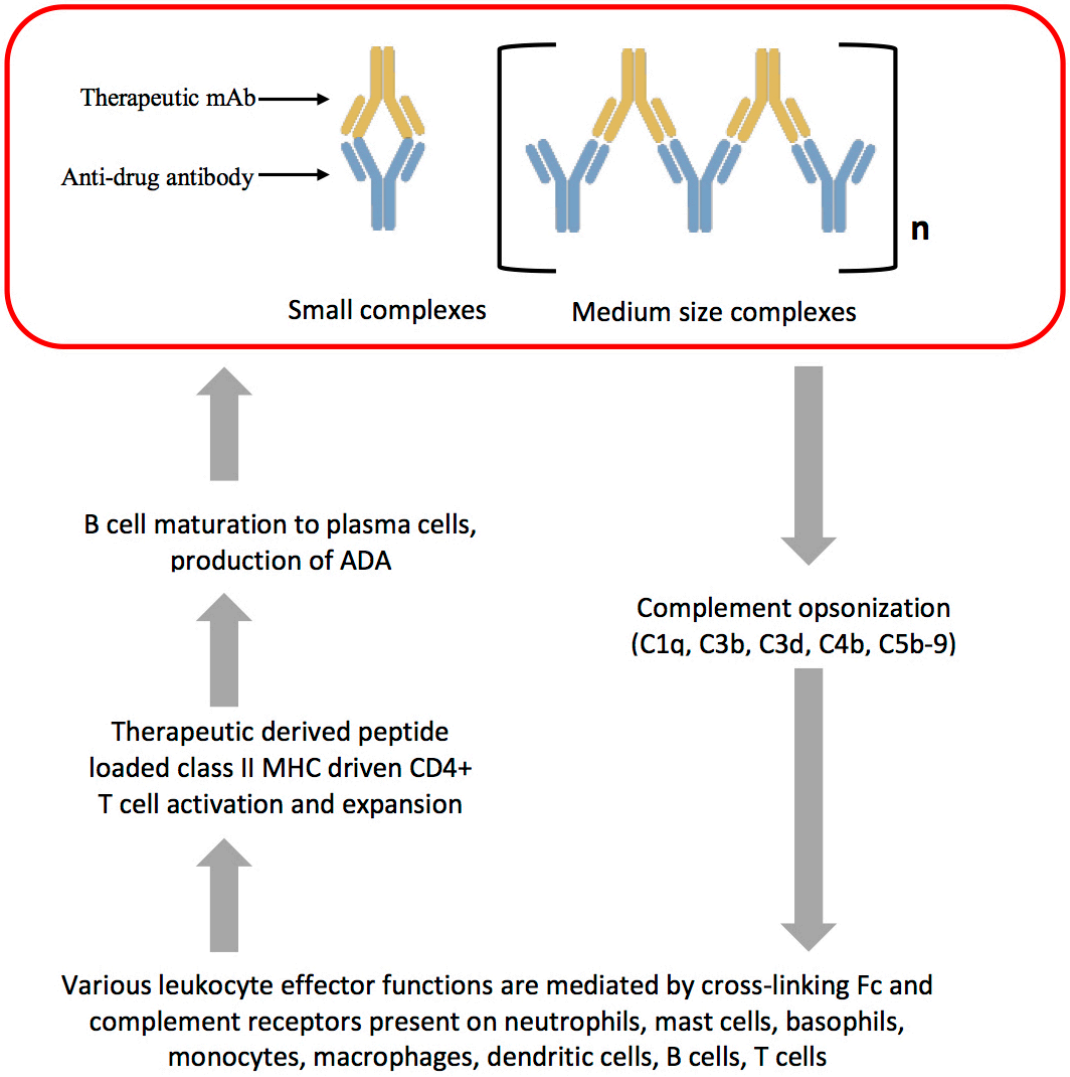

Figure 1.3: The central role of immune complexes formed by therapeutic monoclonal antibodies in the interplay between innate and adaptive arms of the immune system and exacerbation of ADA response. ADA specific to complementary determining regions of a monoclonal therapeutic antibody is used as an example for ease of representing complex formation of varying sizes where *n* represents the number of cross-linked ADA Fc in immune complexes; in reality ADAs are polyclonal with varying specificities. Adapted from Krishna and Nadler (2016).[158]

1.4.2 Methods of Anti-Drug Antibody Detection

Multiple methods have been developed for the detection of therapeutic antibodies and anti-drug antibodies (ADAs). However, as of yet, there is no consensus on the optimal method of detection and measurement of these antibodies due to differences in sensitivity and specificity between techniques.[146] Despite this, there is agreement that these parameters should be measured at 'trough level'. Trough samples are taken prior to the patients next administered dose and thus, represent the lowest serum drug concentration. In contrast to peak levels, trough levels provide a better characterization of patient response by allowing time for the therapeutic antibody to undergo pharmacokinetic and pharmacodynamic processes.[153]

To date, serum concentrations of therapeutic antibodies and their respective ADAs are predominantly measured using an enzyme-linked immunosorbent assay (ELISA).[143] Typically, a bridging ELISA (bELISA) is used to detect ADAs due to superior specificity over conventional ELISA techniques.[144] Bridging ELISAs rely on the binding of free ADAs to solid-phase therapeutic drug which in turn, can be identified using a labelled therapeutic antibody (Figure 1.4). Advantageously, ELISAs are quick, simple and cost-effective, permitting greater clinical applicability compared to alternative methods such as homogeneous shift mobility assays (HSMAs), which require specialist personnel and expensive equipment.[146] However, HSMAs display high sensitivity, a feat that remains a major limitation of ELISA-based methods. During HSMAs, size exclusion high pressure liquid chromatography (SE-HPLC) is employed to distinguish free drug and ADA-drug complexes based on molecular weight differences.[146] Conversely, because ELISAs rely on the binding of free ADAs to solid-phase drug, they are unable to detect ADAs complexed to mAbs.[143] To overcome this, Bourdage et al., described an affinity capture elution (ACE) ELISA-based assay for the detection of anti-drug antibody in the presence of drug. In contrast to previous ELISA methods, the ACE ELISA assay requires initial acid treatment of samples to dissociate ADA-mAb complexes and therefore, diminishes cross-interference from therapeutic antibody. Previously bound ADAs may then be detected through affinity-capture to solid-phase drug, similar to traditional ELISAs (Figure 1.5).[141]

Currently, there are no commercial assays available for the detection of anti-ICI antibodies and little data has been published regarding their prevalence and influence on patient response. The limited data available has been conducted as part of the regulatory application for these drugs, and was performed using a standard bridging ELISA, which as mentioned above bears a number of limitations.[148] Therefore, further immunogenicity investigations are required, especially in real life patients.[147] Developing and validating an affinity capture elution (ACE) assay for detection of ADAs will help us determine the extent to which ADAs occur and alter patient response, ultimately improving our understanding of ICI immunogenicity and optimizing patient care. Additionally, a comparison with a standard bridging ELISA method will provide an insight into the accuracy of these techniques, along with their concordance.

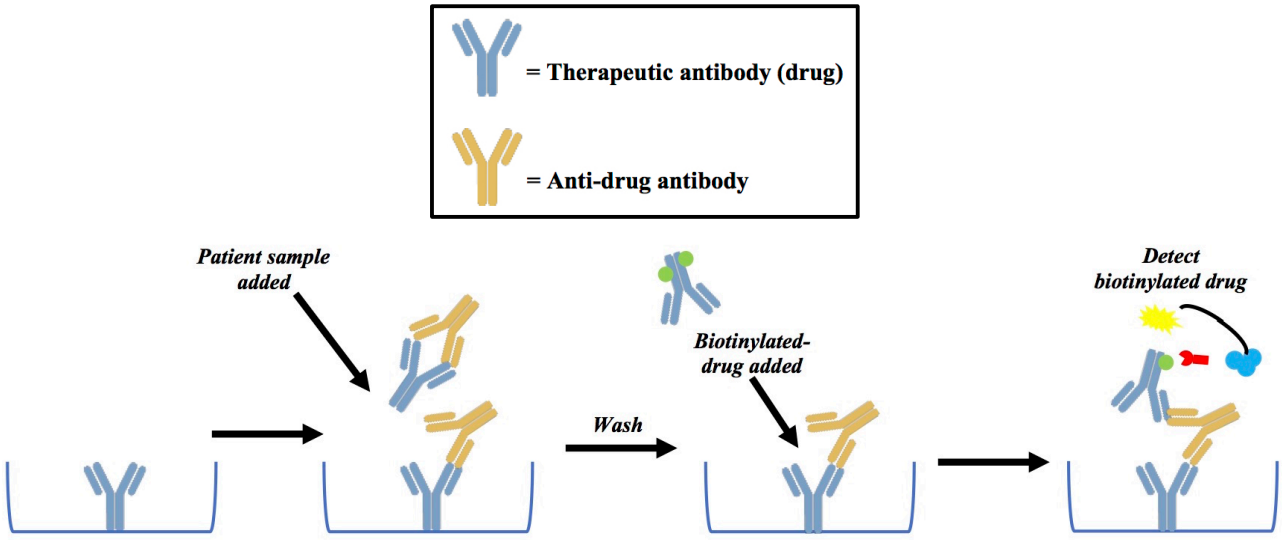

Figure 1.4: Key steps of the bridging ELISA to detect ADAs. ADAs from patient serum bind to therapeutic antibody (drug) coated on an ELISA plate. These ADAs are subsequently detected with therapeutic antibody labelled with biotin. Biotinylation allows for detection of the therapeutic antibody and attached ADA with an enzyme and measurable substrate.

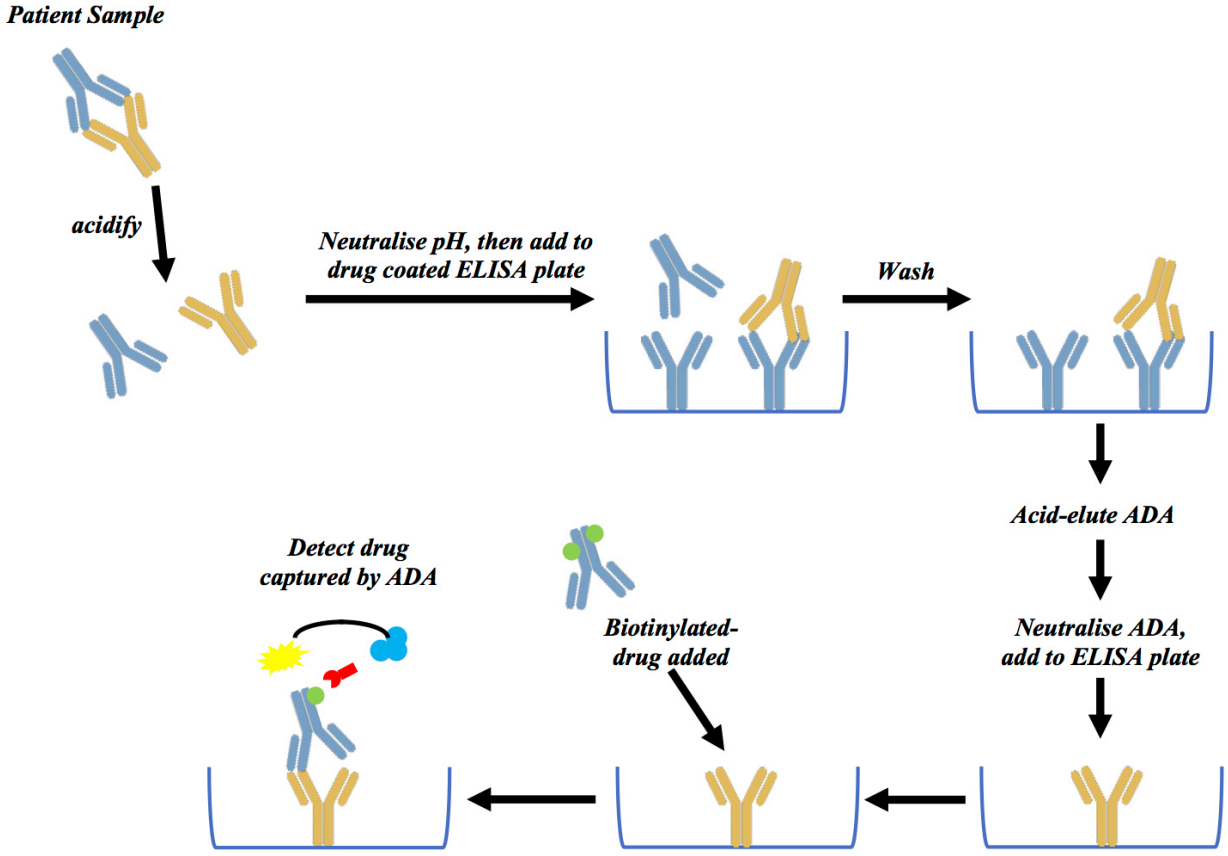

Figure 1.5: Key steps of the affinity capture elute (ACE) ELISA to detect anti-drug antibodies. Firstly, acidification of patient serum dissociates ADA-drug complexes into free drug and free ADA. Following neutralization, ADAs are affinity captured in the in the presence of solid-phase drug. After the removal of free drug by washing, acid-eluted ADA is transferred onto a fresh ELISA plate. Captured ADAs can then be detected through the addition of labelled therapeutic antibody attached to an enzyme and substrate.

1.5 Immune related adverse events (irAEs)

Currently, there are limited guidelines on how to accurately interpret or record irAEs. Common terminology criteria for adverse events (CTCAE) grades events from 1-5 according to severity. Low grade events, grade 1 and 2, represent mild and moderate events, respectively. Whereas, high grade events, grade 3 and 4, describe severe events that are not immediately life-threatening (grade 3) or life-threatening (grade 4). Additionally, grade 5 indicates death related to an adverse event.[79] Systematic reviews have uncovered irAEs are inconsistently and inadequately reported in clinical trials. Notably, consistency in recording incidence, onset and duration of irAEs is lacking.[80,81] Similarly, a review by Michot et al. argued data such as time of onset, reversibility, management, as well as the recording of infrequent or unexpected events not initially thought to be immune related, should be accurately reported. Also highlighted was an absence of consistent terminology throughout the literature, with irAEs referred to as 'drug-related adverse events' or 'events of special interest'.[2] It appears that these inaccuracies may be partly attributed to the difficulty distinguishing between irAEs, non-immune events or typical autoimmune diseases.[33] Paradoxically, inducing an anti-tumour response in patients induces similar responses to those that drive autoimmunity.[82] Therefore, irAEs were initially thought to be similar to the autoimmune diseases that they symptomatically replicate.[83] Now, however, it is believed the two are distinct from one another. For instance, women are more likely to present with autoimmune disease, however, no gender bias has been observed with irAEs.[84] Moreover, age has proven to be a factor in various autoimmune diseases yet the question remains as to whether age has a role in the appearance of irAEs in ICI therapy.[85] Additionally, correlations between identifying factors such as autoantibodies, antigen-specific T cells and MHC haplotypes have not been thoroughly analysed.[82] At present, factors that contribute to the onset of autoimmune-like irAEs seen with immune checkpoint inhibitor therapy remain unclear.

1.5.1 Toxicity profiles

An understanding of ICI toxicity is advancing following widespread treatment of patients outside of clinical trials. Toxicity can potentially present in any tissue forming an expansive clinical spectrum of irAEs as shown in Figure 1.6.[2] Skin toxicities remain the most frequently reported irAE, with grade 1/2 events mainly occurring dermatologically.[86,87] Conversely, grade 3/4 events are more prevalent within the GI tract, and approximately 20% of these events are estimated to be life-threatening. [2,88]

In early clinical trials, CTLA-4 inhibitors and PD-1 inhibitors have been shown to exhibit distinct clinical profiles with regard to both efficacy and toxicity.[10,11] Presumably, these differences

are due to their distinct inhibitory interactions.[47] For example, as seen in Figure 1.7B, a higher incidence of hypothyroidism is associated with PD-1 inhibitors, whereas colitis is more likely to present in anti-CTLA-4 treated patients.[89] PD-1 inhibitors appear to have a generally milder toxicity profile in comparison with CTLA-4 inhibitors. Studies indicate up to 90% of patients undergoing anti-CTLA-4 therapy and 70% of patients undergoing anti-PD-1 therapy experience irAEs.[10,11] A recent meta-analysis reported a higher odds ratio for treatment-related deaths in patients treated with CTLA-4 inhibitors (OR=1.80) compared to patients treated with PD-1/PD-L1 inhibitors (OR=0.63).[18] Despite the risk of serious complications and death, toxicities are usually appropriately managed and reversible, especially if recognised early, and particularly in anti-PD-1 inhibitor therapy where irAEs are predominantly grade 1 or 2 in severity.[2,87]

Notably, variation in toxicity profiles has been observed within checkpoint inhibitor classes in different cancer types. In patients undergoing ICI therapy, it was initially thought vitiligo only presented itself in patients with melanoma.[90] Vitiligo, an autoimmune disorder characterised by depigmentation of the skin has a long recorded relationship with melanoma.[91] The link is thought to be mediated through an immune response towards antigens shared by normal melanocytes and melanoma cells.[92] Unexpectantly, however, two recent case studies reported the first appearances of vitiligo in patients with lung adenocarcinoma and acute myeloid leukaemia following Nivolumab treatment.[93,94] Regardless of an increased use of ICIs in melanoma patients, current literature inclusive of recent case studies indicates vitiligo is more apparent in melanoma patients compared to other cancer types.[95]

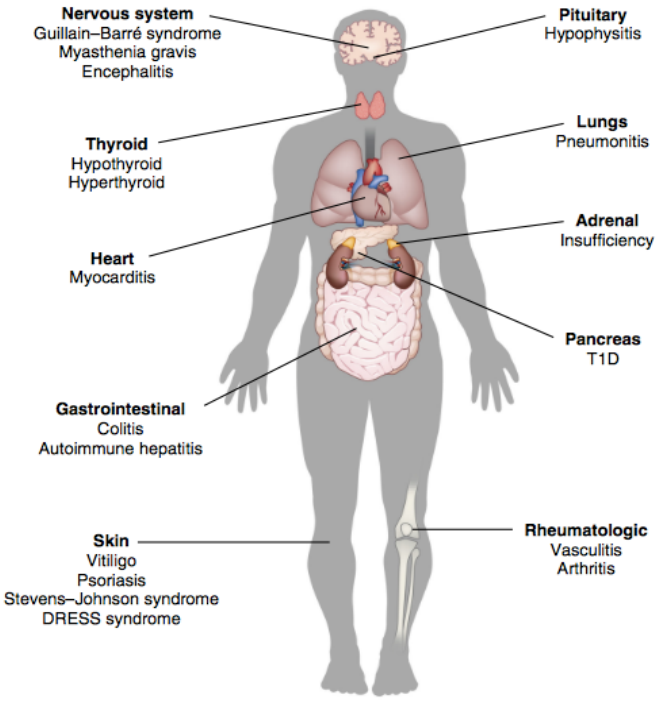

Figure 1.6: Clinical spectrum of irAEs of immune checkpoint inhibitors. T1D, Type 1 Diabetes; DRESS, drug reaction with eosinophilia and systemic symptoms. Adapted from Michot et al. 2016.[2]

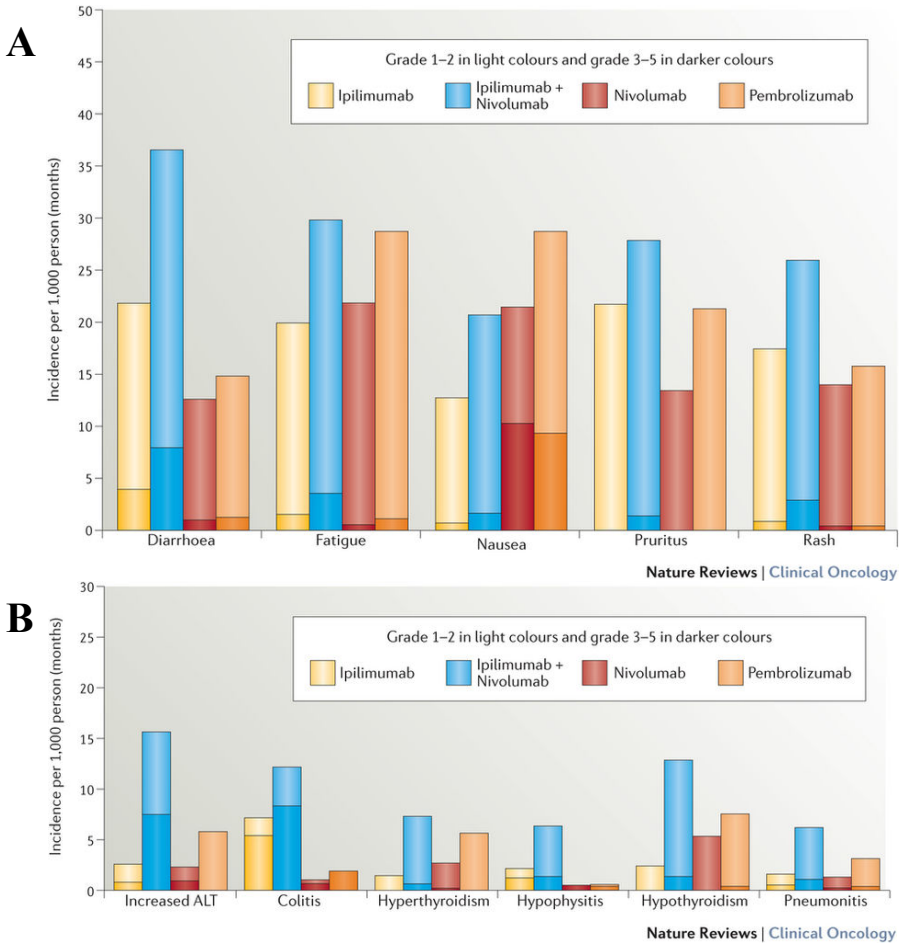

Figure 1.7: Incidence of common (A) and rare (B) immune related adverse events in patients treated with ipilimumab, pembrolizumab, nivolumab or ipilimumab plus nivolumab.
Reprinted from Boutros et al. 2016.[89]

1.5.2 Pathophysiology of irAEs

Despite a general understanding of immune checkpoint inhibition, the exact mechanisms underlying their anti-tumour effects remain unclear. Furthermore, little is known about the mechanisms mediating irAEs. Evidence has shown that some patients do not develop irAEs despite continuous use of ICIs, whilst others experience multiple irAEs throughout treatment.[2,10,11] Additionally, patients may experience toxicity with one type of checkpoint inhibitor but not another.[96,97,98] This may be assumed to be a result of differences in inhibitory targets, but the question remains as to why subtle differences in both efficacy and toxicity are emerging between Nivolumab and Pembrolizumab, two PD-1 inhibitors.[89,99]

1.5.3 Appearance of irAEs and impact on ICI drug efficacy

The literature is mixed with regards to the impact of irAEs of immunotherapy. An association between the development of irAEs and efficacy of ICI therapy has been reported but remains controversial. A link was first described in Attia's (2005) study, which found a correlation between

grade 3/4 irAEs and cancer regression in ipilimumab-treated patients. 36% of patients presenting with a grade 3/4 irAE achieved a response compared to 5% of patients who did not experience any irAEs.[100] With logical reason it has been suggested that the appearance of irAEs is evidence of greater immune system stimulation caused by the immunotherapy. Multiple cutaneous irAEs, including vitiligo and hypopigmentation, are known positive prognostic factors in patients with melanoma.[101,102] Hence, as immunotherapies may induce these irAEs, argument that a greater response occurs in patients presenting with these events is reasonable. A prospective observational study and a small number of retrospective analyses have reported an association between the appearance of cutaneous irAEs and response to anti-PD-1 therapy.[103,104,105] These findings are consistent with previous studies on alternate immunotherapeutic approaches, which noted a correlation between the development of irAEs with improved survival in melanoma patients undergoing interleukin 2 and interferon alfa-2 immunotherapy.[101,106]

In contrast to studies described above, multiple studies have found no evidence of association between the development of toxicity and response to treatment with ICI-therapy.[107,108,109] The reported association of irAEs with anti-tumour responses may be due to lead time bias. Patients who respond to the immunotherapy undergo a longer treatment period and have more time to develop irAEs. Alternatively, those who do not respond or experience disease progression have a shorter treatment period due to a change to an alternative therapy or death.[110] It is important to note that the onset of irAEs is not a prerequisite for response and all of the above studies also found anti-tumour responses in patients who did not experience irAEs. Despite this, the possible association between the appearance of irAEs and improved patient outcome observed in multiple studies should not be dismissed.

1.5.4 Management of irAEs and impact on efficacy

The FDA-approved Risk and Management System guides clinical management of irAEs induced from immune checkpoint inhibitor therapy. Algorithms generally suggest supportive measures for low grade irAEs, whereas temporary or permanent discontinuation of the drug is recommended for high grade irAEs.[111] Additionally, administration of systemic corticosteroids and immunosuppressants may be advised in high grade events.[2,111] Logically, it has been suggested that the dose and treatment regime must be closely managed to avoid a decrease in efficacy of the immunotherapy. Yet, current literature indicates adjunctive treatment with immunosuppressants does not influence the response to either CTLA-4 inhibitors or PD-1 inhibitors.[100,109] Studies into the use of ICIs in organ transplant patients may help determine the impact of immunosuppressants in treatment, however, thus far, limited studies including this population have been published.

1.6 Biomarkers

Numerous studies have investigated biomarkers that may predict response to therapy. However, little focus has been extended to biomarkers which could predict the development of toxicity. Furthermore, no studies have been published that investigate biomarkers of toxicity in patients treated with PD-1/PD-L1 inhibitors.

1.6.1 Biomarkers predictive of response to treatment

In order to select patients most likely to benefit from treatment, previous literature has focused on identifying biomarkers that predict efficacy of ICI treatment. A focal point involves the intratumoural expression of PD-L1, and a number of studies have reported its association with enhanced clinical efficacy.[11,112,113,114,115] An initial study conducted on nivolumab-treated patients found 9 out of 25 patients (36%) with PD-L1 positive tumour cells had an objective response, whereas no patients with PD-L1 negative tumour cells responded.[11] Similarly, a number of recent studies found a greater response rate in patients with PD-L1 positive tumours. In contrast, however, these studies also observed objective responses in PD-L1 negative patients.[112,113,114,115] These results contribute to the growing body of evidence that PD-L1 expression may indicate an increased likelihood of response to anti-PD-1/PD-L1 treatment, but is not sufficient to exclude PD-L1 negative patients from therapy. As more evidence comes to light, the role of PD-L1 expression as a predictive biomarker has been questioned for multiple reasons. Most importantly, the validity of the data is flawed due to differences in staining methods, antibodies used, cut-off points for determining positivity and interpretation of the resulting data.[116,117,118] All of which, have led to controversy surrounding the role of PD-L1 expression as a predictive biomarker.

However, PD-L1 expression by immunohistochemistry is FDA-approved to select patients for treatment with pembrolizumab. This success may arise from the markedly increased expression of PD-L1 in NSCLC cells compared to other tumour types, including melanoma cells. Kluger et al., showed that expression of PD-L1 not only varies between tumour types but also varies within patients between stromal cells and tumour cells.[116] Thus, these variables need to be considered in future evaluations of PD-L1 as a predictive biomarker. Another PD-1 ligand, PD-L2, has been less studied, but recently associated with a positive clinical response in patients with head and neck squamous cell carcinoma (HNSCC)[154]. Beside the expression of PD-1 ligands, other biomarkers suggested to predict patient response to treatment include white blood cell counts, mutational or neoantigen burden and the microbiome.[119,120,121,122,123] It has also been suggested that a combined biomarker approach may be more effective in predicting response to ICI treatment.[124]

A barrier for biomarker discovery is the atypical response patterns observed in ICI-therapy. Unconventionally, tumours treated with ICIs may exhibit 'pseudoprogression', in which an initial apparent increase in size of a tumour is followed by regression.[156] This phenomenom has led to the question of whether conventional response evaluation criteria in solid tumours (RECIST), a standardized criteria that a number of studies involving ICI-treated patients use, is representative of a true response to immunotherapy.[135] Traditionally, significant tumour progression on therapy has been considered indicative of treatment failure, but, an estimated 10-15% of metastatic melanoma patients treated with immune checkpoint inhibitors experience pseudoprogression.[155] Retrospective findings of pseudoprogression, along with clinical observations in patients undergoing immunotherapy, have prompted the development of immune-related response criteria (irRC) and immune-related response evaluation criteria in solid tumours (iRECIST).[156,157] The intended benefit of these developments is to better categorize patients with unconventional response patterns. Even so, regulatory authorities have not yet approved these guidelines for use in clinical trials and RECIST remains a standard assessment for tumour response to ICI-therapy. With the exception of PD-L1 in NSCLC, no single or combination biomarkers have been discovered to clearly predict response to ICI therapy.

1.6.2 Biomarkers predictive of toxicity

Several studies have described biomarkers which may be predictive of toxicity in patients undergoing checkpoint inhibitor therapy. Shahabi's (2013) study, showed two neutrophil-activation markers, CD177 and CEACAM1 are promising markers of toxicity.[125] In this study, gene expression profiling on whole blood samples from melanoma patients treated with ipilimumab revealed an increased expression of these neutrophil-associated genes (along with multiple other immunologically relevant genes) was associated with gastrointestinal (GI) toxicity. The possible role of neutrophils in GI toxicity is backed by an earlier study, which observed an association between colonic inflammation prior to initiation of treatment and diarrhoea. Histopathologic examination of patient colonic biopsies revealed infiltration of lamina propria by neutrophils, presence of crypt abscesses and glandular destructions or erosions in patients at higher risk of GI toxicity. Additionally, this study also found an increase in neutrophil-derived fecal calprotein, a biomarker of bowel inflammation, was associated with diarrhoea.[126]

Moreover, the appearance of GI irAEs has been linked with elevated interleukin-17 (IL-17) serum levels. Two studies evaluating IL-17 levels in ipilimumab-treated melanoma patients have reported an association with colitis.[127,128] The first, Callahan et al, (2011) reported no significant difference in baseline IL-17 serum levels between patients with or without colitis, but significantly higher IL-17 serum levels in patients with colitis at weeks 7 and 12.[127] In contrast, a second study

conducted by Tarhini et al., (2015) found a significantly higher IL-17 serum level at baseline in patients who not only developed colitis but any grade 3 irAEs.[128] It is unknown whether IL-17 levels at weeks 7 and 12 correlated with the study of Callahan et al., as blood serum samples were not collected during these time points and thus, were unable to be analysed. Since a functional link exists between IL-17 and eosinophils, it is not surprising a further correlation between absolute and relative eosinophil counts has also been implicated in the occurrence of irAEs.[129]

All of the above studies focus on potential biomarkers that may predict toxicity of GI irAEs. There is limited evidence regarding predictive biomarkers of toxicity in other tissues. Furthermore, all of these studies described patients treated with ipilimumab, an anti-CTLA-4 inhibitor that evidently has a different mechanism of action to PD-1 inhibitors. No studies have been published describing biomarkers which reliably predict the development of toxicity in patients treated with PD-1 inhibitors. Therefore, the question remains as to whether suggested biomarkers predictive of toxicity in patients treated with CTLA-4 inhibitors may predict toxicity in patients treated with PD-1 inhibitors. Additionally, whether similar biomarkers can predict toxicities in different organs is another question yet to be answered.

In contrast to studies described above, an alternative approach to identify biomarkers of patient response is the investigation of anti-drug antibody production. A previous link has been described between the production of ADAs towards mAb therapeutics and an increased incidence of adverse events.[140,142] However, the precise underlying mechanisms by which mAb therapeutics induce the production of ADAs and subsequently, how these anti-drug antibodies confer toxicity is yet to be confirmed. At present, toxicological effects are believed to originate from the formation of immune complexes between ADAs and therapeutic mAbs.[137,158] Interaction of these complexes with Fc and complement receptors mediates leucocyte effector functions giving rise to the toxicological effects observed in previous studies.[158] Theoretically, similar immunogenic consequences could be observed in patients treated with nivolumab or pembrolizumab. Thus, one understudied approach that may predict the development of toxicity in patients undergoing anti-PD-1 therapy, is the analysis of anti-drug antibodies. It remains unknown as to whether the formation of anti-drug antibodies in patients undergoing ICI-therapy increases their risk of toxicity, a question we aim to address in this study.

1.6.3 Role in treatment

The importance of early diagnosis, clinical monitoring and successful management of irAEs is highlighted throughout the literature.[2,130,131] In order to optimise patient care and minimise damage, it is essential to identify patients at increased risk of toxicity. Although, irAEs have been associated with a greater efficacy of ICI-therapy, the risk of debilitating, serious and life-threatening events

can outweigh the benefits received from treatment. A recent review by Hamanishi et al., described the issues of PD-1/PD-L1 blockade in cancer treatment, emphasizing the identification of biomarkers is a crucial step in advancing the application of ICIs.[132] They also noted 'a great deal of fundamental, exploratory research remains to be done on areas such as predictive biomarkers for therapeutic efficacy and adverse drug reactions.[132] As a whole, the literature is in agreement with this statement, documenting a need for biomarkers predictive of toxicity. Identifying and validating predictive biomarkers will help distinguish patients suitable for ICI therapy, avoiding unnecessary toxicity and health-care costs.[133]

1.7 Conclusion

In summary, maximising the anti-tumour effect whilst minimising toxicities remains a crucial goal in achieving the greatest clinical benefit from immune checkpoint inhibitor therapy. Problematically however, increasing stimulation of the patient's immune system to increase the anti-tumour effect simultaneously increases the risk of irAEs, in what can be described as a 'double-edged sword' effect. In upcoming years, completion of clinical trials of existing and newly developed ICIs will increase clinical usage. Yet, several questions remain regarding the underlying mechanisms mediating the anti-tumour effects and irAEs induced by this class of agents. Furthermore, no biomarkers have been identified to predict the development of toxicity to ICIs, particularly in treatment with PD-1 inhibitors. Therefore, to advance current understanding, the overall aim of this exploratory study is to investigate clinicopathological indicators, anti-drug antibody levels and circulating markers of inflammation that may predict patient outcome.

1.8 Study objectives and thesis outline

The primary objective of this exploratory study is to improve our understanding of patient response to immune checkpoint inhibitors. Thus, this research will be conducted in three parts to investigate biomarkers of response (toxicity and efficacy) to anti-PD-1 treatment. The specific objectives are:

1. To examine clinicopathological indicators of response, patient data will be retrospectively reviewed and analysed for associations between patient statistics and response.
2. To examine whether anti-drug antibodies decrease efficacy or increase toxicity, therapeutic antibody and anti-drug antibody levels will be measured in patient serum and analysed for associations with patient outcome including development of immune-related toxicities, response and survival.

3. To examine circulating markers of inflammation that may predict patient response, patient serum will be analysed for blood biomarkers using a commercially available antibody array and ELISA.

Due to time constraints, objective 3 was not completed as part of this project. However, it will be completed outside of this project's time frame to build on preliminary data produced in objectives 1 and 2. Therefore, the ensuing chapters describe objectives 1 and 2. Chapter 2 describes the retrospective review of patient data to explore clinicopathological biomarkers of patient outcome, whereas chapter 3 describes the use of an in-house developed ELISA to determine if patients treated with pembrolizumab produce anti-drug antibodies and their association with drug levels and patient outcome.

1.9 Hypotheses

Based on previous literature we hypothesized that clinicopathological characteristics and levels of circulating inflammatory markers will vary between patients with different outcomes to treatment. Additionally, patients at a higher risk of developing autoimmune disease will exhibit an increased risk of developing toxicities. We also hypothesized that patients who develop anti-drug antibodies experience a decrease in treatment efficacy and/or an increase in toxicity. Also, patients who produce ADAs and experience decreased efficacy of treatment will have lower circulating drug levels.

Chapter 2

2 Retrospective review

2.1 Introduction

As with all newly approved therapeutics, the use of immune checkpoint inhibitors outside of clinical trials is anticipated to generate additional data regarding the efficacy and toxicity of these agents. Of particular interest, is the use of ICIs in patients that fail to satisfy inclusion criteria of initial clinical trials. Following FDA-approval of nivolumab and pembrolizumab in 2014, PHARMAC began funding anti-PD-1 treatment for patients in NZ with advanced melanoma (stage III and IV) from the 1st of July 2016.[150, 161] In clinical trials, immune-related adverse events observably display similar characteristics to autoimmune conditions, however, their underlying etiology is yet to be determined.[83] Demographic characteristics are known contributors to the onset of autoimmune disease, but, thus far, it is unknown as to whether these variables factor in the development of irAEs. In particular, women and elderly individuals represent high-risk populations for autoimmune conditions.[84,85] Owing to this increased risk and the observed similarities of autoimmune conditions with irAEs, we sought to determine whether demographic characteristics may predict patient outcome. To date, no demographic characteristics have been associated with patient response or survival. Therefore, we focused on their association with toxicity. Specifically, we hypothesise that patients at an increased risk of developing autoimmune disease also have an increased risk of developing irAEs during ICI-therapy.

Alternatively, it has been suggested that blood parameters may act as preferential biomarkers for response to ICI-therapy.[123,124] The underlying mechanism of checkpoint inhibition is unknown, but, it is clear that white blood cells, play an important role. Neutrophils, lymphocytes and eosinophils have each been implicated in patient response and the development of irAEs, but, the interplay between blood parameters and patient outcome is yet to be confirmed.[123,125,129] To investigate the possible use of blood biomarkers in ICI-therapy, this chapter analysed the complete blood counts of ICI-treated patients and their association with patient outcome.

An exploration into the relationship of patient clinicopathological variables with patient outcome is of clinical relevance to determine patients suitable for ICI therapy. Exploration in real-life patients is particularly warranted given the lack of published literature investigating ICIs outside of regulatory clinical trials. Ideal biomarkers will select patients for treatment based on an increased likelihood of responding to treatment or a decreased risk of developing severe toxicity. Ultimately, the discovery of such biomarkers will prevent a subset of patients from undertaking an expensive, ineffective treatment that carries potential life-threatening toxicity. Therefore, the

primary aim of this chapter was to determine whether clinicopathological characteristics predict patient outcome (in terms of patient response, survival and the development of toxicity) and examine their potentiality as a biomarker(s) for ICI therapy in clinical practice.

2.2 Materials and Methods

2.2.1 Study Participants

This study involved patients treated for stage III or IV metastatic melanoma with nivolumab or pembrolizumab by Canterbury Regional Cancer and Haematology services. Patients were eligible for inclusion if they had a confirmed diagnosis of stage III or IV metastatic melanoma and received at least one dose of nivolumab or pembrolizumab at Christchurch Hospital, New Zealand. Patients unable to provide informed consent or under 18 years of age were excluded from the study. All participants gave written informed consent for use of available clinicopathological data for scientific purposes. Ethical approval for this study was obtained from the Southern Health and Disability Ethics Committee of New Zealand (approval 16/NTB/139).

2.2.2 Treatment Regimen

Patients were treated at the Oncology unit in Christchurch Hospital, New Zealand, with nivolumab or pembrolizumab as a monotherapy for metastatic melanoma according to national PHARMAC guidelines. Patients were treated as per standard of care treatment. Nivolumab was administered at a dose of 3mg/kg intravenously over 60 minutes every 2 weeks (Q2W). Pembrolizumab was administered at a dose of 2mg/kg intravenously over 30 minutes every 3 weeks (Q3W). Treatment was continued until: (1) disease progression according to RECIST version 1.1 by radiographic assessment, primarily computed tomography (CT) scan; and/or (2) unacceptable toxicity as assessed by the practicing clinician.

2.2.3 Data collection

With approval from the Southern Health and Disability Ethics Committee, New Zealand, eligible patients were identified for review from the Christchurch Hospital Medical Oncology database. Patients treated with nivolumab (Q2W) or pembrolizumab (Q3W) as a monotherapy for metastatic melanoma (stage III or IV) between 1st July 2016 to 30th June 2017 were retrospectively reviewed using electronic medical records and clinician notes. Collection of data was completed after appropriate training and all information was collected and collated under the supervision of Dr. Matthew Strother (Medical Oncologist and Clinical Pharmacologist). The following clinical data was collected: patient demographics, medical history, disease status, prior treatment, current

therapeutic regimen, response to treatment, laboratory blood values and toxicity events. The data cut-off point was 30th June 2017. All patients were followed up until death, or data collection endpoint.

2.2.4 Haematological Parameters

Haematological parameters were derived from laboratory blood tests routinely performed at the Canterbury District Health Board (CDHB) Canterbury Health Labs or Southern District Health Board (SDHB) Southern Community Laboratories. Resulting complete blood counts were obtained from patient electronic medical records. Complete blood counts were recorded for patients prior to treatment (baseline) and throughout treatment, until 28 days after discontinuation of treatment or death. The normal range of complete blood count values in healthy individuals were obtained from the Christchurch Hospital Medical Oncology database. Baseline complete blood counts were used to calculate white blood cell ratios. Specifically, neutrophil to lymphocyte ratio (NLR) was calculated by division of neutrophil counts by lymphocyte counts and lymphocyte to monocyte ratio (LMR) was calculated by division of lymphocyte counts by monocyte counts.

2.2.5 Response Evaluation

To assess response to treatment, patients underwent CT scanning prior to treatment (baseline), followed by restaging CT scanning at 12 week intervals after first infusion of drug. Initial baseline assessments and subsequent response to treatment was determined by a medical practitioner according to Response Evaluation Criteria in Solid Tumours (RECIST) version 1.1. Responders were classified as patients that met RECIST criteria for complete or partial response. Non-responders were classified as patients that met RECIST criteria for stable or progressive disease. Time to progression was calculated from the date of starting treatment to the earliest CT scan showing disease progression by RECIST criteria or death. The best overall response was defined as the best response recorded from the start of treatment until disease progression.

2.2.6 Toxicity Assessment

Data collection of adverse events from electronic medical records and clinician notes was completed for events occurring throughout treatment until 28 days after cessation of treatment or until commencement of subsequent alternative treatment or death. Data collected included the date the event was reported, clinical presentation, grade and treatment plan. The severity of events was graded according to National Cancer Institute Common Terminology Criteria for Adverse Events, version 4.0 (CTCAE v4.0). Grading was completed by a medical practitioner at time of event

reporting, otherwise, grades were retrospectively assigned by the investigator based on description of events by the practicing clinician and CTCAE v4.0. IrAEs were defined as adverse events of unknown cause with a potential immunologic basis, associated with drug exposure.

2.2.7 Statistical analysis

All analyses were carried out using Microsoft Excel version 15.38 and GraphPad Prism version 6.0 (San Diego, CA, USA). Descriptive statistics were used to summarize clinicopathological data including patient demographics and treatment characteristics. Data was summarized as n (%) for categorical variables and as median (range) for continuous variables. The Chi-square test was applied to categorical variables to determine any significant differences between groups. Unpaired t-tests were used to compare continuous variables. Kaplan-Meier curves were used to estimate progression-free survival. Log-rank test was used for comparison of progression-free survival among variable factors. All statistical tests were two-sided and p values <0.05 were considered statistically significant.

2.3 Results

2.3.1 Patient Population

2.3.1.1 Baseline Demographics

Between 1st July 2016 to 30th June 2017, thirty-two patients were treated with nivolumab or pembrolizumab as a monotherapy for metastatic melanoma (stage III or IV) at Christchurch Hospital, New Zealand. A detailed listing of clinicopathological characteristics of these patients is presented in Table 2.1. The median age at data cut-off point was 70 years (range, 30-86 years), with male predominance (72%). Patients were primarily New Zealand European in ethnicity (91%). Twenty-six (81%) patients had a stage IV melanoma, and brain metastases were present in nine (28%) patients.

2.3.1.2 Medical History

On retrospective review, 26 (81%) patients had pre-existing comorbidities. Three patients (9%) had a previously diagnosed autoimmune condition upon starting treatment, namely psoriasis, rheumatoid arthritis or multiple sclerosis. Fourteen (44%) patients had inflammatory conditions. The most common inflammatory condition was gastro-esophageal reflux disease (GORD) occurring in 6 patients, followed by gout (3 patients), pseudogout (2 patients) and asthma (2 patients). Extrinsic allergic alveolitis (hypersensitivity pneumonitis) was present in one patient. Nearly a third

of patients (31%) had a previous non-melanoma cancer. 50% these patients (5 out of 10) had non-melanoma skin cancers, presenting as squamous and basal cell carcinomas. Bladder, blood, cervical and prostate cancer represented the other cancer types recorded for this patient cohort. Over a third of patients (34%) had cardiovascular conditions. Coronary artery disease was reported for 3 patients, with a further 7 patients noting a history of myocardial infarctions, supraventricular tachycardia, angina or transient ischemic attacks. Other notable comorbidities of this patient cohort included, but were not limited to, chronic kidney disease, epilepsy, obesity, dementia and type 2 diabetes mellitus.

2.3.1.3 Therapeutic Regimen

At the beginning of the study, 17 (53%) patients had received prior therapy for metastatic melanoma in the form of radiotherapy, immunotherapy and/or targeted therapy. Radiotherapy was the most common pre-treatment (15 patients), followed by immunotherapy (3 patients) and targeted therapy (1 patient). All 3 patients who received prior immunotherapy underwent treatment with immune checkpoint inhibitors before commencement of the study period. Patients received ICI-therapy through privately funded treatment or as part of a clinical trial. One patient was treated with pembrolizumab monotherapy, one patient underwent sequential ipilimumab and pembrolizumab monotherapy and one patient received a combination therapy of ipilimumab and pembrolizumab. Irrespective of surgical intervention, over half (53%) of patients had received at least one prior therapy and two patients had received two forms of prior therapy. In the current study, 21 patients (66%) received nivolumab and 10 patients (32%) received pembrolizumab. One patient received both PD-1 inhibitors sequentially. A median of five treatment cycles per patient was administered and the maximum number of cycles administered to a patient during the study period was 26. As of the data collection endpoint, treatment with nivolumab or pembrolizumab was ongoing in 16 patients (50%).

Table 2.1: Summary of the clinicopathological data for metastatic melanoma patients used in this retrospective study

Patient characteristic	Total (n = 32)
Age (years), median (range)	70 (30-86)
Sex	
Female	9 (28%)
Male	23 (72%)
Ethnicity	
New Zealand European (Pakeha)	29 (91%)

European, not further defined	2 (6/%)
Unknown	1 (3%)
Body mass index, median (range)	26.6 (18.1-44.2)
Clinical stage	
III	6 (19%)
IV	26 (81%)
Brain metastases	
Yes	9 (28%)
No	23 (72%)
Anti-PD-1 monotherapy	
Pembrolizumab	10 (31%)
Nivolumab	21 (66%)
Pembrolizumab/Nivolumab	1 (3%)
No. of cycles, median (range)	5 (1-26)
Prior therapy	
Chemotherapy	0 (0%)
Targeted therapy (BRAF+MEK inhibitor)	1 (3%)
Immunotherapy	3 (9%)
Radiotherapy	15 (47%)
No. prior therapy	
0	15 (47%)
1	15 (47%)
2	2 (6%)
Comorbidities	
Autoimmune condition	3 (9%)
Inflammatory condition	14 (44%)
Cardiovascular disease	11 (34%)
Non-melanoma cancer	10 (31%)
Any	26 (81%)
Overall status at last follow-up	
Alive	29 (91%)
Deceased	3 (9%)

Values are number and percentage unless otherwise stated

2.3.2 Haematological Parameters

2.3.2.1 Baseline Values

Over half of all patients reviewed had at least one complete blood count (CBC) abnormality prior to treatment (18 patients, 56%). A detailed summary of complete blood count values for patients included in this study is shown in Table 2.2. Baseline eosinophil and basophil counts were unavailable for analysis for three patients and 21 patients, respectively.

Table 2.2: Complete blood count values of patients included in this study

Haematological Parameter	Normal Range	Total (n = 32)	
		Range	Mean
Red cell parameters			
Haemoglobin (g/L)	115-175	99-166	132.5
Haematocrit (%)	0.35-0.52	0.31-0.49	0.41
Mean corpuscular volume (fL)	80-99	77-112	89.6
Mean cell haemoglobin (pg)	27-33	23-36	29.3
White cell parameters			
White cell count ($x10^9$/L)	4-11	3.4-12.3	7.1
Neutrophils ($x10^9$/L)	1.9-7.5	2.4-9.1	4.8
Lymphocytes ($x10^9$/L)	1-4	0.36-3.6	1.45
Monocytes ($x10^9$/L)	0.2-1	0.1-1.3	0.6
Eosinophils ($x10^9$/L)[a]	0.0-0.5	0.0-0.5	0.19
Basophils ($x10^9$/L)[b]	0.0-0.20	0.0-0.23	0.09
Other parameters			
Platelets	150-400	134-474	294.9

Ranges include male and female data
[a] data missing from three patients
[b] data missing from 21 patients

2.3.3 Efficacy of treatment

At data cut-off point, response assessment to anti-PD-1 treatment was available for 27 patients. 5 patients were unevaluable for response as they were yet to undergo their first restaging scan. Of the evaluable 27 patients, 11 patients experienced disease progression, 12 had a partial response to treatment and 4 patients exhibited stable disease as a best overall response, according to RECIST criteria (Table 2.3). One responder exhibited pseudo-progression, in which progressive disease was observed at the first restaging scan, followed by resolution of the progressive disease in a

subsequent scan. The progression-free survival curve of all patients included in this study is shown in Figure 2.1. The median PFS of all patients was 6 months. At last follow-up, 29 patients (91%) remained alive and no deaths were drug related.

Table 2.3: Best overall response of patients reviewed

Best Overall Response	Total (n = 32)
Partial response	12 (37.5%)
Stable disease	4 (12.5%)
Progressive disease	11 (34%)
Response not evaluated	5 (16%)

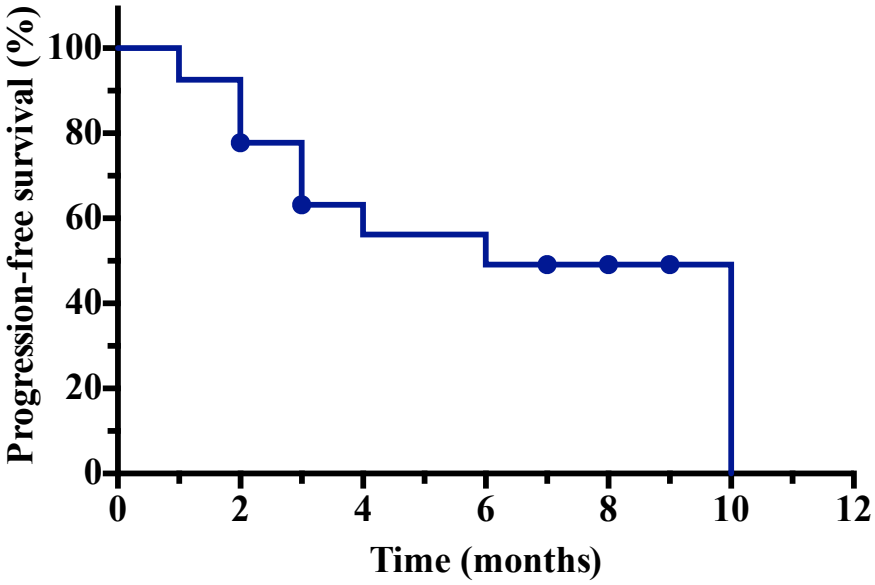

Figure 2.1: Progression-free survival curve of all patients included in this study (n=32).

2.3.4 Toxicity

2.3.4.1 Overview

IrAEs of any grade were observed in 29 (91%) patients. The most commonly reported events included fatigue, rash, dyspnea and constipation, as shown in Table 2.4. Events affecting the skin were most frequently observed, with 41% of all patients reviewed experiencing dermatological effects. The pulmonary and gastrointestinal system were the second and third most adversely affected organ systems with 34% and 25% of patients experiencing pulmonary and GI events, respectively. Events of the endocrine system were less frequently reported, affecting 19% of all patients. The percentage of organ-specific events affecting our patient cohort is shown in Figure 2.2. During the study period, 19 (59%) patients presented with irAEs affecting two or more organs,

either concurrently or sequentially. The majority of events were low-grade. High-grade events were infrequently observed, with 1 patient experiencing a grade 3 skin rash, another requiring hospitalization due to pneumonitis and one experiencing severe fatigue.

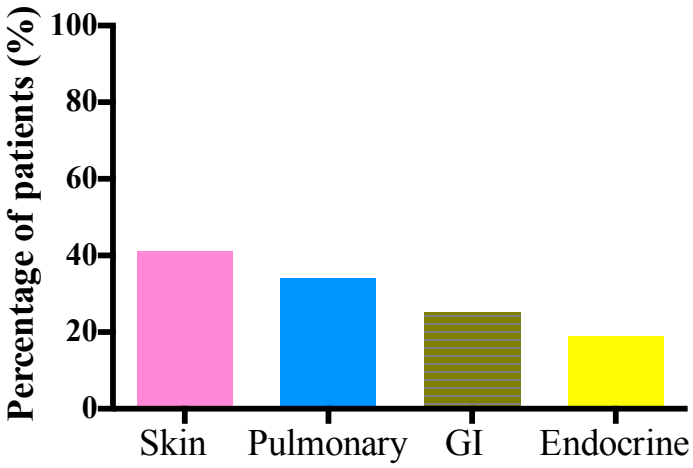

Figure 2.2: Distribution of irAEs by organ. The percentage of patients affected by irAEs (all grades) across different organ systems.

Table 2.4: Incidence of common immune related adverse events in all patients (N=32). Percentages represent the occurrence of an event in all patients including patients who experienced multiple irAEs.

Event	Any Grade	Grade 3-4
Fatigue	22 (69%)	1 (3%)
Rash	11 (34%)	1 (3%)
Dyspnea	8 (25%)	0 (0%)
Constipation	7 (22%)	0 (0%)
Cough	6 (19%)	0 (0%)
Nausea	6 (19%)	0 (0%)
Pruritus	6 (19%)	0 (0%)
Diarrhea	4 (13%)	0 (0%)

2.3.4.2 Management of irAEs

IrAEs were generally managed with supportive care, withholding of treatment or intervention with corticosteroids. Of the 29 patients who experienced an irAE, seven (24%) required treatment with steroids. Appearance of a skin rash was the predominant reason for steroid initiation (5 patients), followed by pneumonitis (one patient), cough (one patient) and a hypersensitivity reaction (one patient). It should be noted the patient who was prescribed steroids for pneumonitis was the same patient who was initiated on steroids for a hypersensitivity reaction. In this patient, these events

occurred sequentially and separate steroids were prescribed for each event. Prednisone was most frequently prescribed, followed by hydrocortisone and clobetasone. Other interventions included aqueous creams, thyroid hormone, carbimazole, laxatives, antihistamines, anti-emetics, analgesics, and nonsteroidal anti-inflammatory drugs (NSAIDs).

Four (13%) patients had treatment withheld due to irAEs. One patient delayed cycle 23 by 1 week due to grade 1-2 transaminitis. No further intervention was required and the patient was able to re-commence ICI-therapy. One patient withheld, restarted and later switched anti-PD-1 therapy from nivolumab to pembrolizumab, due to a grade 3 skin rash. In this case, the initial use of clobetasone and hydrocortisone, in conjunction with treatment discontinuation resulted in a significant improvement in rash. A grade 3 rash recurred upon restarting of nivolumab, prompting the prescription of high dose prednisone (80mg). High dose steroid use and the replacement of nivolumab with pembrolizumab resulted in complete resolution of the rash and the ability to continue treating the patient with ICIs. One patient withheld cycle 2 due to esophagitis and gastritis, but was not restarted on treatment due to disease progression. One patient was hospitalized due to pembrolizumab-induced pneumonitis, resulting in a delay of cycle 3 of treatment by 2 weeks. Following ICI-therapy delay and intervention with corticosteroids, the patient recovered from pneumonitis showing no further symptoms during the study period. In all four cases, withholding of the therapeutic agent and/or intervention with steroids was sufficient to allow the patient to continue ICI-therapy.

2.3.4.3 Dermatological events

Skin was the most adversely affected organ with 13 (41%) patients experiencing dermatological irAEs. Events were primarily low-grade and high-grade skin irAEs were not observed, with the exception of one grade 3 skin rash. Clinically, the dermatological events observed in this study were rash, pruritus and psoriasis, descending in order of prevalence. Eleven (34%) patients developed a skin rash during treatment, typically following the first or second infusion. The median time to rash presentation was 3 weeks (range, 1-13 weeks) and maculopapular erythematous rashes were commonly described. Pruritus was observed concurrently with rash in five patients, but also manifested in one patient without rash toxicity. A detailed summary of the demographic and clinical characteristics of patients who presented with rash during this study are shown in Table 2.5. One case of psoriasis was recorded for a patient with pre-existing psoriasis who experienced an exacerbation of the condition upon commencing pembrolizumab, followed by symptom stabilization. No further dermatological events were reported in this study.

Table 2.5: Demographic and clinical characteristics of patients presenting with rash toxicity during anti-PD-1 treatment

Case	Age	Sex	Grade	Week of onset, (cycle of anti-PD-1 therapy)	Clinical presentation	Location	Cessation of drug	Steroid intervention
1	83	m	1	3 (cycle 2)	macular erythematous, desquamation, eczematous	arms, legs, torso	no	yes
2	82	f	1-2	1 (cycle 1)	maculopapular erythematous, excoriation	arms, trunk (back)	no	yes
3	37	f	1-2	4 (cycle 3)	not reported	not reported	no	no
4	59	m	1	10 (cycle 3)	macular	forearms	no	no
5	71	m	1	6 (cycle 2)	not reported	legs	no	no
6	69	m	1	1 (cycle 1)	macular erythematous	arms, legs, torso, trunk (back), scalp	no	yes
7	69	m	1	1 (cycle 1)	not reported	trunk (chest)	no	yes
8	75	m	1-2	2 (cycle 1)	macular papular	arms	no	no
9	68	m	3	5 (cycle 3)	maculopapular erythematous, dry desquamation	arms, legs, torso, back, neck, face	yes	yes
10	73	m	1-2	13 (cycle 4)	mild petechial	legs	no	no
11	39	f	1-2	3 (cycle 2)	not reported	forearms	no	no

2.3.4.4 Respiratory events

Respiratory events were the second most reported, with irAEs reported in 11 (29%) patients. Following commencement of anti-PD-1 therapy, dyspnea (shortness of breath) was reported by eight (25%) patients. Six (19%) patients developed a cough throughout therapy and, of these patients, one developed a productive cough. All dyspnea and cough events were low grade. One patient presented to hospital with a confirmed case of high grade pembrolizumab-induced pneumonitis. Two additional patients developed chest infections however, it was unclear as to whether the pneumonitis event in these patients was an infective process or secondary to anti-PD-1 therapy.

2.3.4.5 Gastrointestinal events

25% of patients experienced irAEs affecting the gastrointestinal tract. During treatment, four patients reported diarrhea. In all four cases, diarrhea was low grade (<6 diarrheal bowel movements per day over baseline). Two additional patients reported diarrhea during the study period but these events were deemed not related to treatment, due to existence prior to therapy or causation of symptoms through the use of anti-constipation medication. Six patients (19%) experienced nausea throughout treatment and one patient exhibited grade 2 vomiting (4 episodes within 24hrs). A further patient, not experiencing nausea, reported one episode of vomiting. Seven patients (22%) reported constipation throughout treatment, however, in two of these patients the constipation event was deemed not related to treatment, instead, attributed to the use of opiate analgesics. Three patients (9%) developed abdominal pain upon starting treatment.

2.3.4.6 Endocrine events

In this study, endocrine events reported were low grade and generally related to the thyroid gland. Alteration of thyroid gland function was detected in six (19%) patients. Specifically, hypothyroidism was detected in three patients and hyperthyroidism in one patient. Subclinical hypothyroidism and hyperthyroidism was identified in two patients with low or high levels of thyroid-stimulating hormone, respectively. One case of adrenal insufficiency was reported in a patient. However, the insufficiency was attributed to cessation of hydrocortisone, rather than an effect of ICI-therapy.

2.3.4.7 Other events

Of all the adverse events described in this study, fatigue was the most frequently reported, affecting 22 (69%) patients. Less common events reported included infusion-related reactions, musculoskeletal events and hematological events. Two (6%) patients experienced hypersensitivity reactions, both attributed to pembrolizumab infusion. In the first case, a 59-year-old male experienced a reaction during cycle 3 of treatment requiring immediate intervention with intravenous steroids and antihistamines. The second case, which required no intervention, involved a 57-year-old female who experienced a mild reaction following cycle 1 of treatment. Pain was reported for 14 (44%) patients throughout treatment, but, typically preceded ICI-therapy or was attributed to disease progression. However, three patients specifically reported joint pain (arthralgia) in response to anti-PD-1 therapy. In one patient, arthralgia was a recurring symptom of underlying arthritis. Another patient also experienced a flare in a pre-existing inflammatory condition, noting an increase in gout-related symptoms. At the end of the study period, one patient with elevated creatine levels and one patient with abnormal liver function tests were noted to have potential drug-related nephritis or hepatitis, respectively. Other effects reported included a loss of appetite (five patients), swelling (three patients) and anemia (two patients).

2.3.5 Association of clinicopathological variables, efficacy and toxicity

No statistically significant differences were found between clinicopathological variables and patient outcome including patient response, survival and development of toxicity. In particular, no statistically significant differences in progression-free survival were found between males and females or between patients over the age of 70 and patients under the age of 70 (Figure 2.3). There was also no statistically significant difference in progression-free survival between patients with different white blood cell ratios, as shown in Figure 2.4. Additionally, no significant difference in progression-free survival was found between patients who developed a rash and patients who did not develop a rash during ICI-therapy (Figure 2.5). Similarly, there was no statistically significant differences in baseline haematological values of responders versus non-responders (Figure 2.6) and patients who developed a rash versus patients who did not develop a rash (Figure 2.7) No statistically significant differences were found between clinicopathological variables and the development of toxicity in any organ or of any grade. No further statistical analysis was possible due to small sample size.

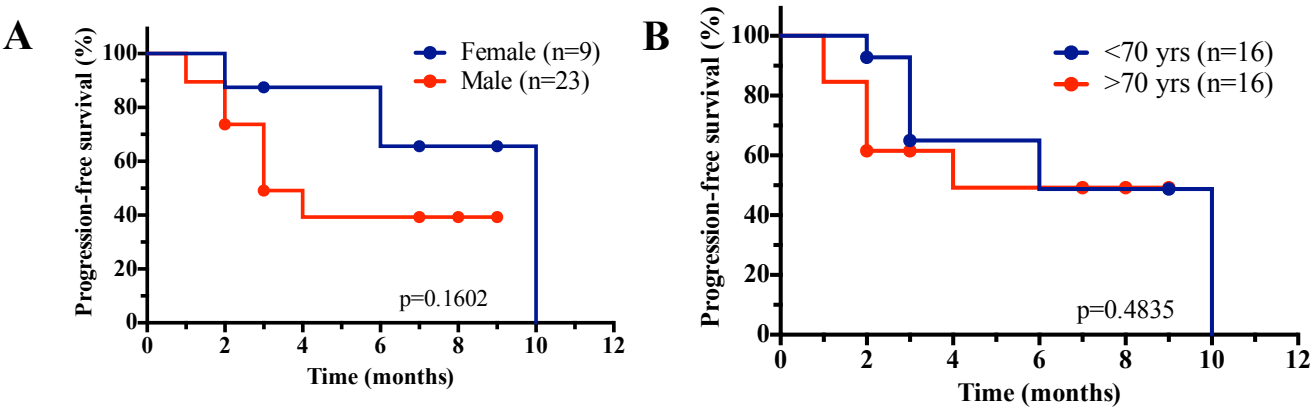

Figure 2.3: Progression-free survival of patients included in this study stratified by demographic variables. Kaplan-Meier curves for patients stratified by gender (A) and age (B).

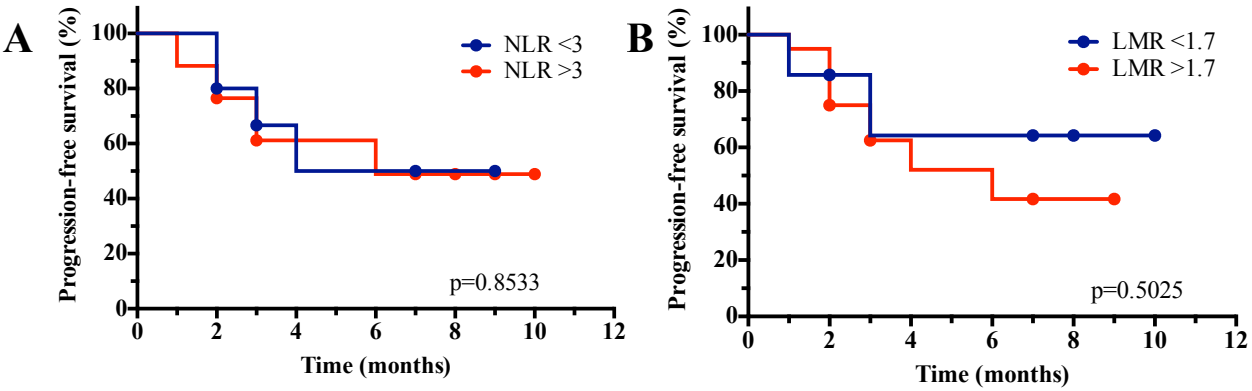

Figure 2.4: Progression-free survival of patients included in this study stratified by baseline white blood cell ratios. Kaplan-Meier curves for patients stratified by neutrophil to lymphocyte ratio (NLR) (A) and lymphocyte to monocyte ratio (LMR) (B).

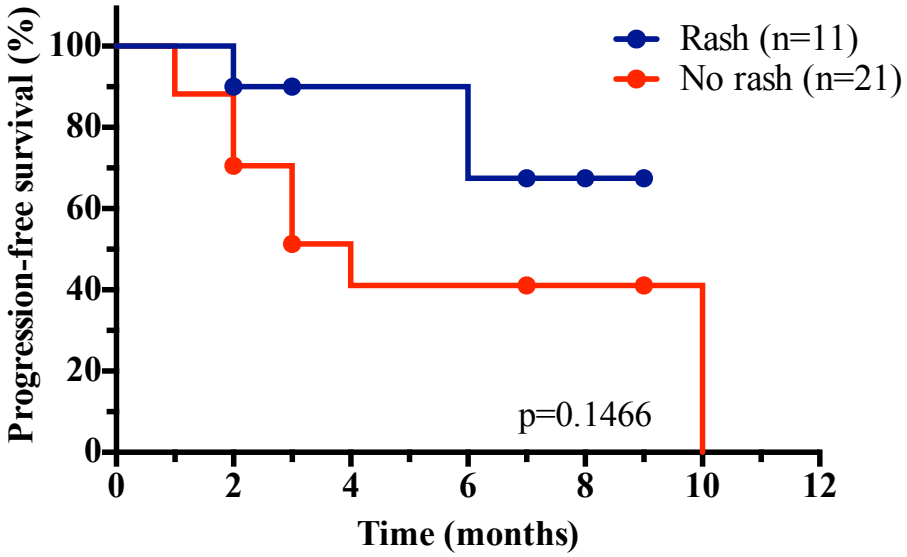

Figure 2.5: Progression-free survival of patients included in this study stratified by rash toxicity.

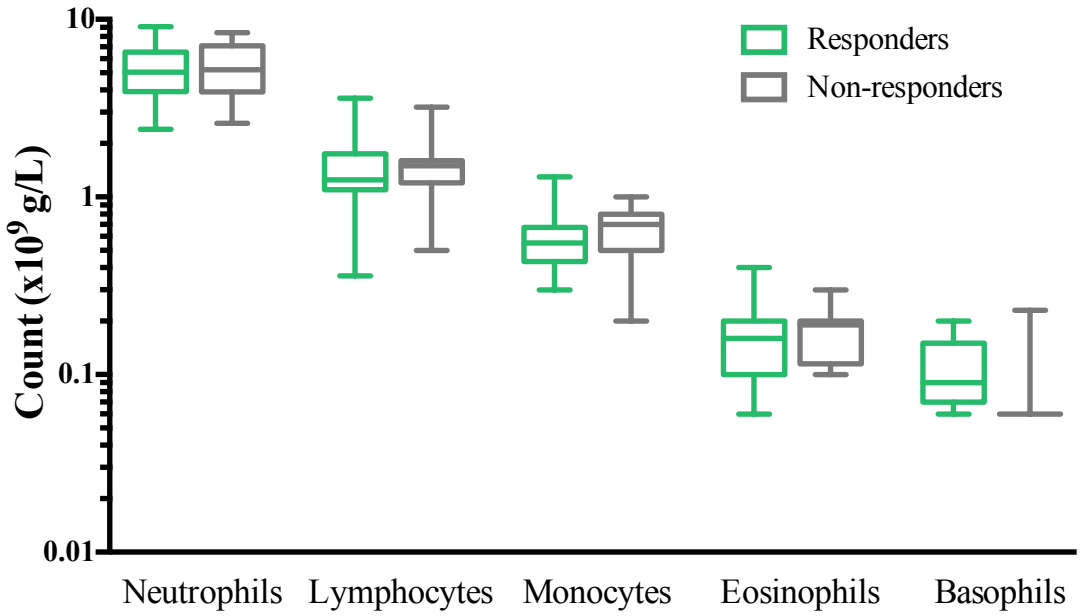

Figure 2.6: Baseline peripheral white blood cell counts of responders and non-responders. Selected baseline peripheral white blood cell counts of responders (n=12) and non-responders (n=16) are presented as box and whisker plots. Whiskers indicate minimum and maximum values for each group and the edge of the box represents the twenty-fifth and seventy fifth quartiles. Median values are indicated by a line inside the box. No significant differences between white blood cell counts was found between responders and non-responders at baseline.

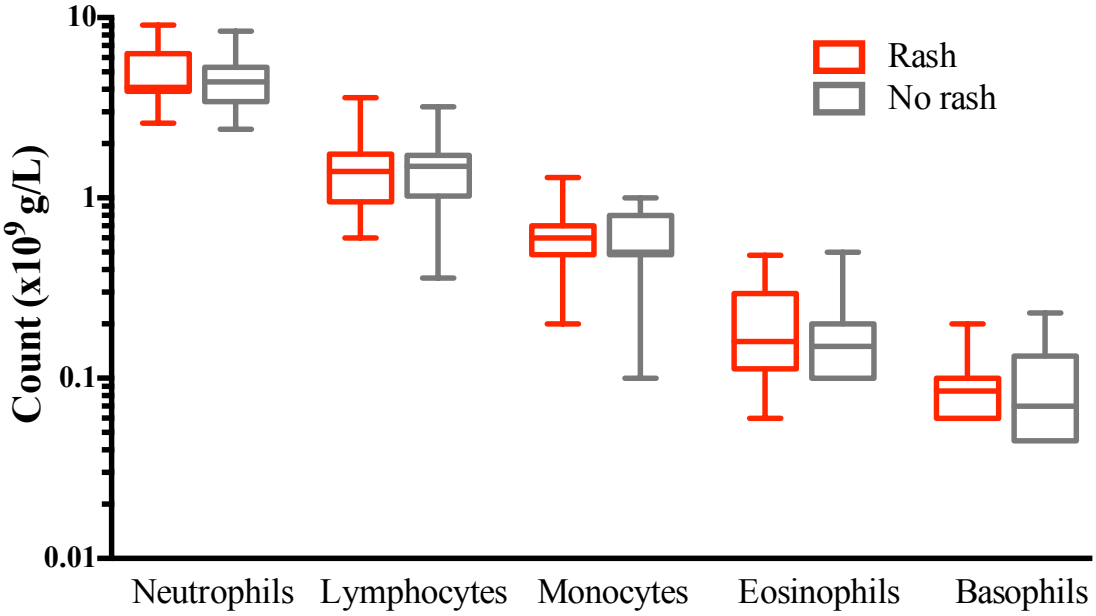

Figure 2.7: Baseline peripheral white blood cell counts of patients who developed rash versus patients who did not develop rash. Selected white blood cell counts of patients who developed a rash (n=11) and patients who did not develop a rash (n=21) are presented as box and whisker plots. Whiskers indicate minimum and maximum values for each group and the edge of the box represents the twenty-fifth and seventy fifth quartiles. Median values are indicated by a line inside the box. No significant differences between white blood cell counts were found between patients who developed a rash and patients who did not develop a rash.

2.4 Discussion

The production of immune-related adverse events and low efficacy in a subset of patients poses a significant downfall to ICI-therapy. To prevent a proportion of patients exposed to an ineffective and potentially dangerous therapy, there is an unmet need for biomarkers that will select appropriate patients for treatment. This chapter reviewed patients treated with anti-PD-1 monotherapy to identify clinicopathological markers associated with patient outcome. Specifically, this chapter sought to determine the potential for clinicopathological biomarkers to reliably predict patient survival, response and/or development of toxicity.

The only exclusion criteria for this retrospective review was the exclusion of patients under the age of 18. Clinical trials completed as part of the regulatory approval process of nivolumab or pembrolizumab excluded patients with one or more of the following characteristics; brain metastases, a history of autoimmune disease, prior therapy (particularly with antibodies that modulate T-cell function), conditions requiring immunosuppressive medications and inadequate hematologic, hepatic or renal function. Early clinical trials also excluded patients with significant comorbidities and/or prior malignancies.[10,11,19,20,21] If this exclusion criteria were re-applied to our study population, 72% of patients would be ineligible for participation in early trials of anti-PD-1 agents. The high percentage of patients unable to fulfil inclusion criteria of early trials may be, to some extent, attributed to the higher median age of patients in this study (70 years).[10,11] Unsurprisingly, as patients age, the risk of developing significant comorbidities, impaired organ function and/or acquiring conditions that require immunosuppressive medications increases, rendering these patients ineligible for various clinical trials.[159] Yet, oncologists will regularly encounter these patients in real life clinical settings, highlighting a need for safe and effective treatment options. To explore how patients who would be ineligible under clinical trial protocols respond to ICI-therapy, all patients were included in the current study, given they were above 18 years of age.

Data is beginning to emerge from studies examining the efficacy and toxicity of anti-PD-1 agents in specialist patient populations, such as those with organ transplants and various autoimmune diseases.[160,162,164] Patients with autoimmune conditions are typically excluded from clinical trials of ICIs, due to safety concerns. Treatment and management of autoimmune disease requires suppression of the immune system, an opposite effect to that induced by immune checkpoint inhibitors.[1,160] Thus, due to their mechanism of action, ICIs may exacerbate underlying autoimmune conditions and increase the risk of treatment-induced irAEs. Unfortunately, the prevalence of autoimmune disease is increasing, particularly in industrialized countries, affecting a large number

of individuals who may require oncological treatment with ICIs.[163] Only recently has data been published reporting the efficacy and safety of anti-PD-1 agents in patients specifically with autoimmune disease.[160,164] A systematic review conducted by Abdel-Wahab et al, (2016) found that 28.9% of patients with underlying autoimmune disease experienced flares of their condition upon commencement of ICI-therapy.[160] In the current study, a low number of participants had an underlying autoimmune disease (n=3) and, in line with previous literature, 1 in 3 patients had an exacerbation of underlying autoimmune disease. Importantly, no high-grade irAEs were observed in this subset, demonstrating that ICIs may safely be used without exacerbation of disease or risk of developing serious toxicities. The efficacy of ICI treatment in this subset is yet to be established, with a best overall response of stable disease observed in two patients in this study. However, due to the small number of patients with autoimmune disease in this study, no conclusions can be drawn. Studies investigating a greater number of participants are required to determine the risk-benefit profile of ICI-therapy in this specialist population.

It is well established that females and individuals of an older age have a higher risk of developing autoimmune disease.[84,85] Interestingly, the three cases of patients with autoimmune disease in this study, described two males and one female of a typically younger age (36, 39 and 58 years old). Nonetheless, due to the observed symptomatic similarities between autoimmune disease and irAEs, we hypothesized that risk factors of autoimmune disease would also factor in the development of irAEs during ICI-therapy. In opposition to this hypothesis, our study found no significant differences between the development of irAEs (of any grade, or in any organ) and patient age or gender, further, supporting the idea that the underlying pathophysiology between the two conditions is different. Our patient cohort was predominately male (72%) and of an older age (median=70). This is to be expected as, in New Zealand, the risk of developing melanoma increases with age and males are more commonly affected. Data from the Ministry of Health NZ (2013-2015), show melanoma was most frequently diagnosed in male individuals, particularly those aged 70-74.[165] However, the gender imbalance observed in this study, is markedly different from previous data. Of the patients diagnosed with melanoma in NZ in 2015, 55% were male and 45% were female.[165] This may be explained by a trend that has been observed overseas, describing a faster increase in the incidence of melanoma in men than women.[166] However, it is most likely attributed to chance.

Our study showed no significant differences in patient response or survival between females and males or patients under the age of 70 versus patients over the age of 70. Given the rise of melanoma diagnoses with age, the efficacy of immune checkpoint inhibitors in elderly patients is of particular clinical relevance. No conclusions can be drawn from our study, due to limited numbers,

but our data is in line with previous literature, which indicates ICIs are an equally efficacious treatment option for geriatric melanoma patients.[167]

In this study consisting of patients deemed ineligible or eligible for earlier clinical trials (n=32), 44% responded to treatment. This figure is in line with previous studies, albeit slightly higher.[10,11] Patients who responded to treatment according to standard RECIST criteria, appeared to maintain their response throughout the study period. During the study period, only one patient who achieved a response went on to experience disease progression. Caution should be exercised however, due to a relatively short study period (12 months). Nonetheless, this supports previous studies, which indicate anti-PD-1 agents induce sustained responses, a characteristic which confers an advantage over conventional treatment options. It has been suggested that the 'memory' of the immune system is responsible for the formation of long-lasting responses.[168] As these agents have been newly approved, the potential maximal response duration is yet to be determined. A further question exists as to whether these agents may be tantamount to cures for some individuals. If so, studies into the appropriate duration of treatment should be conducted to determine whether patients could cease therapy to prevent the occurrence of serious irAEs.

Our study reported a high incidence in the development of immune-related adverse events, observing events in 91% of patients. Previous studies indicate that up to 70% of patients undergoing anti-PD-1 treatment experience irAEs.[2] This markedly increased incidence may be due a number of factors. Firstly, it is well established that the incidence of irAEs in clinical trials of immune checkpoint inhibitors is underreported. Some studies only report irAEs that occur above a certain threshold, ranging from above >1% and 5-10%.[80] More importantly, a limitation in this field of research is the absence of a uniform definition of an irAE. No standardized criteria exists to determine whether adverse events are of an immune-related origin or are the result of alternate aetiology.[2] In our study, we classified irAEs as adverse events of unknown cause with a potential immunologic basis, associated with drug exposure. However, discrepancies in definition can make comparison of incidences between studies difficult. Additionally, the FDA warn that, due to differing conditions of conducted clinical trials, adverse event rates may not reflect the rates observed in general practice.[75,76] The use of these agents in real-life patients has been suggested to contribute to an increased prevalence of irAEs in general practice.[81] An alternative hypothesis to the increased incidence of irAEs observed in this study, is that patients normally excluded from clinical trials are at greater risk of developing toxicity. However, this possibility requires further investigation in a larger cohort to determine if such a relationship exists.

Despite differences in overall incidence, the type and frequency of irAEs observed in this study are in line with patterns of occurrence in previous studies. Events commonly reported in earlier studies such as fatigue, rash, and constipation, largely affected our patient cohort.[2,10,11,21] In particular, skin rashes were the most frequently reported organ-specific event. A trend amongst previous literature is the sequential appearance of events affecting different organ systems. Skin irAEs tend to appear early during therapy, followed by gastrointestinal events and lastly by endocrine and hepatic events.[2,134] A similar trend was observed in the present study. Nine of the 13 patients who presented with dermatological events reported rash, pruritus or psoriasis as their first occurring event. Additionally, gastrointestinal, endocrine or hepatic symptoms were generally described later in therapy. The majority of events occur within the first 12 weeks of treatment (before an initial restaging scan), but the appearance of irAEs has been reported up to 1 year after starting anti-PD-1 treatment.[2,181] Even though our data collection period spanned over 1 year, the majority of patients reviewed started treatment within this period, rather than at the beginning of the study. Owing to this pattern of appearance, a longer study duration or a follow up study of these patients would be interesting to observe whether events typically occurring later in treatment appear.

Consistent with previous studies, uncommon events were less prevalent. Furthermore, no rare irAEs such as Gullian-Barre syndrome, Stevens-Johnson syndrome or aseptic meningitis were observed in our study.[2] Presumably, these absences are due to the design of this exploratory study which lacks the power to detect less common events, particularly rare events occurring in less than 1% of patients. In order to increase the power of future studies to detect rare events, a larger sample size could be analysed. The severity of events in our study also followed a similar pattern to published literature. Grade 3-4 events have been documented for 12-20% of patients treated with nivolumab or pembrolizumab and three patients (9%) experienced severe irAEs in the present study.[169] The slightly lower incidence may be attributed to a small sample size, but may denote an increased awareness of potential irAEs and their appropriate management by physicians. As more cohort or case studies are published, physicians become increasingly aware of the potential irAEs that may occur and importantly, learn how they may be effectively treated or managed before their severity increases.

A controversial link has been reported between the development of irAEs (particularly dermatological events) and response to ICI-therapy.[103,104,105] To explore this theory, we compared the progression-free survival of patients who developed a rash during therapy to those who did not develop a rash in our patient cohort. We observed no significant difference between these two subgroups ($p=0.1466$) but, interestingly, noted a trend towards improved PFS of patients who developed a rash. Caution must be exercised when interpreting this result due to the small number

of patients in each subgroup, specifically 11 patients who developed rash versus 21 patients who did not. While a statistically significant association between rash and patient response to anti-PD-1 therapy has been described previously in multiple studies, these findings need to be validated in studies incorporating a larger number of study participants. Although an interesting trend towards improved progression-free survival was seen in patients who developed a rash, our findings can neither confirm nor negate this proposed link.

Additionally, pseudo-progression was observed in one patient in this study. Initial enlargement of a tumour in patients with unconventional response patterns is thought to be mediated by lymphocyte infiltration into the tumour, rather than tumour cell proliferation.[156] Interestingly, when monitoring the lymphocyte counts of this particular patient in our study, an increase in circulating lymphocytes from baseline occurred at the time of apparent disease progression. Whereas, before the ensuing CT scan, which showed regression of the tumour, the patient's lymphocyte count had decreased. In this study however, we did not investigate the tumour microenvironment directly and therefore, it is unknown as to whether an increase in circulating lymphocytes correlated with increased tumour infiltration of lymphocytes. In future studies, an investigation into the association of blood parameters with pseudoprogression, rather than conventional response patterns may assist in the development of response criteria and differentiation between true progression and pseudoprogression. However, as to whether the patient case in this study had an increased infiltration of lymphocytes into the tumour is unknown. A biopsy of the tumour with subsequent immunohistochemistry could have been applied to investigate blood parameters within the tumour microenvironment. Preliminary data from a recent study found patients who experienced pseudoprogression tended to be younger in age than those who did not.[170] This finding is yet to be validated, but serves as an indication that clinicopathological factors may contribute to pseudoprogression. The present study reports one case of pseudoprogression in an 80-year-old female and thus, is unable to contribute to this theory, but it is important to recognize that pseudoprogression may also occur in elderly patients.

Research investigating peripheral blood biomarkers has proposed an association between various haematological parameters and patient response, survival or the development of toxicity.[123,124,125,129] In particular, a lower neutrophil to lymphocyte ratio (NLR) has been associated with a shorter progression-free survival.[171] Conversely, our study found no significant difference in PFS between patients with a NLR above three and patients with a NLR below three. Furthermore, a study published earlier this month (October 2017), reported the first analysis of the lymphocyte to monocyte ratio (LMR) in patients treated with immune checkpoint inhibitors.[172] In this study, a

baseline LMR of less than 1.7 was associated with improved PFS and overall survival in metastatic melanoma patients treated with pembrolizumab. The present study found no significant difference in PFS, but similarly noted a trend towards improved PFS in patients with a LMR below 1.7. Furthermore, in contrast to previous studies our study detected no significant differences at baseline between haematological parameters of responders versus non-responders. These results may be due to small numbers preventing the ability of significant associations to be detected, however, highlights the idea that patients with different baseline blood values may respond the same to therapy or vice versa. Additionally, this data supports the idea that circulating blood biomarkers previously associated with patient outcome, may not possess the specificity or reliability to select patients for treatment. Peripheral blood markers are preferentially sought after in biomarker studies due to their ease of accessibility and routine measurement in clinical practice. However, in the instance of ICI-therapy blood markers within the tumour microenvironment, an avenue we did not explore in this study, might prove more useful in predicting patient outcome.

Potential limitations of the present study were its retrospective design and inclusion of a small number of patients (n=32) from a single institution. Due to the study design, analysis was subject to biases in data recording and collection that inherently result from investigations of a retrospective nature. It is possible only significant or noticeable events were reported by patients or clinicians, leaving less serious or unnoticed events unaccounted for. Despite these potential drawbacks, a high incidence of low-grade irAEs affecting a wide range of organs were observed in the current study, demonstrating thorough event recording by managing physicians. The collection of data from a single institution may in fact be advantageous, capturing a holistic view of anti-PD-1 treated patients and a greater likelihood of consistent reporting of clinical events and their management. Additionally, due to the recent approval of the first anti-PD-1 agents, nivolumab and pembrolizumab in 2014, these agents represent a new area of research.[75,76] Besides large regulatory clinical trials, many studies to date have been conducted for exploratory purposes and involve samples sizes similar to that of the current study.[177,179,180] For example, a recently published study by Sanmamed et al, (2017) analysed serum IL-8 levels in 29 melanoma patients treated with nivolumab or pembrolizumab.[177]

In summary, this chapter assessed the potentiality of clinicopathological variables as biomarkers of patient outcome to ICI-therapy. We did not identify any statistically significant associations between clinicopathological variables and patient outcome, due to a relatively small sample number. Thus, no candidate markers of a clinicopathological origin were identified to potentially predict patient outcome with regard to survival, response or the development of toxicity. Despite

this, results of this chapter suggest that patients ineligible for early clinical trials of immune checkpoint inhibitors may be effectively treated in general practice with nivolumab or pembrolizumab, but perhaps carry a greater risk of developing toxicity. Further research in a larger cohort is required to investigate this observation and determine the risk-benefit profile of specialist patient populations.

Chapter 3

3 Analysis of Drug Concentration and Anti-Drug Antibodies

3.1 Introduction

As with all therapeutic antibodies, pembrolizumab and nivolumab can potentially produce undesirable immunogenic responses upon administration, leading to the production of anti-drug antibodies.[136,137] More importantly, however, these anti-drug antibodies have been associated with decreased efficacy and/or increased toxicity for a range of therapeutic mAbs.[140,142,144,152] Based on this association, we questioned whether anti-drug antibodies are generated in patients undergoing ICI-therapy. If so, their production may predict how patients respond to treatment and assist in guiding therapeutic regimens. Thus far, limited studies have examined the prevalence of anti-drug antibodies in patients receiving immune checkpoint inhibitors and even fewer have investigated their association with patient outcome. The largest studies, conducted as part of the regulatory approval of these drugs, use a conventional bridging ELISA technique that has considerable limitations with respect to the detection of ADAs (see section 1.4.2 in Chapter 1).[148]

To address this limitation, the Haematology research group (HRG) based at the University of Otago (Christchurch, NZ), developed a modified ELISA for the analysis of drug and anti-drug levels. Last year, they published a study describing the use of this method in patients with rheumatoid arthritis and inflammatory bowel disease receiving anti-TNF mAbs.[178] The ELISA developed by the HRG follows similar principles to the affinity capture elute (ACE) assay described by Bourdage et al, (2007). In that assay, the patient serum undergoes an initial acidification step in order to break up any existing drug-ADA complexes and then captures all ADA present.[141] The ELISA method used in the current study is analogous to the ELISA developed by the HRG. However, instead of applying the assay to patients receiving mAbs for autoimmune disease, it has been applied to patients receiving anti-PD-1 mAbs (nivolumab or pembrolizumab) for the treatment of metastatic melanoma.

The specific aim of this chapter was to examine whether the production of anti-drug antibodies in patients undergoing ICI-therapy alters patient outcome. If so, detection of anti-drug antibodies may act as a predictive biomarker during ICI-therapy, allowing physicians to guide and manage treatment more effectively in general practice. Based on previous studies of therapeutic mAbs, we hypothesized patients who produce anti-drug antibodies exhibit decreased efficacy of treatment and/or an increased risk of toxicity. Additionally, we hypothesized that patients who produce ADAs have a lower circulating drug concentration. To investigate this, an in-house developed ELISA was used for analysis of drug concentrations and anti-drug antibody levels in sera

from patients undergoing immune checkpoint therapy. Resulting data was analysed for associations with patient outcome, including the development of immune-related toxicities, response and survival. To our knowledge, this study is the first to test these parameters in patients undergoing ICI-therapy using an acid dissociation ELISA method.

3.2 Materials and Methods

3.2.1 Study Participants

This study involved patients treated for stage III or IV metastatic melanoma with nivolumab or pembrolizumab by Canterbury Regional Cancer and Haematology services. Patients were eligible for inclusion if they had a confirmed diagnosis of stage III or IV metastatic melanoma and received at least one dose of nivolumab or pembrolizumab at Christchurch Hospital, New Zealand. Treatment was administered according to national PHARMAC guidelines, as described in chapter 2, section 2.2.2. Patients under 18 years of age or with a haemoglobin level lower than 90 g/L were excluded from blood sample analysis. Patients unable to provide informed consent or participating in a competing clinical trial were also excluded. Ethical approval for this study was obtained from the Southern Health and Disability Ethics Committee of New Zealand (approval 16/NTB/139) and all participants gave written informed consent for use of biomaterials and available clinicopathological data for scientific purposes. Patient age, gender, response to treatment, appearance of irAEs was collected from electronic medical records and clinician notes. Collection of data was completed after appropriate training and all information was collected and collated under the supervision of Dr. Matthew Strother.

3.2.2 Patient Samples

Serum samples were collected from patients after informed consent and approval from the Southern Health and Disability Ethics Committee (New Zealand). Sera from patients receiving nivolumab or pembrolizumab monotherapy for metastatic melanoma (stage III or IV) was collected at trough time points, immediately prior to the patients next scheduled infusion, as shown in Figure 3.1. Samples were collected from patients at multiple infusion points throughout treatment. Collection of samples from infusion 1 was not mandatory but, following initiation of sample collection, sera was collected at consecutive infusion time points. Drug naïve control sera were obtained from a panel of stored diagnostic Immunology laboratory samples that had undergone testing for tissue transglutaminase IgA. Serum samples were stored at -80℃. 20µl aliquots were separated and stored at -20℃ until use. Samples were assayed for therapeutic antibody concentration and levels of anti-ICI antibodies. When assayed, two replicates of each individual sample were analysed.

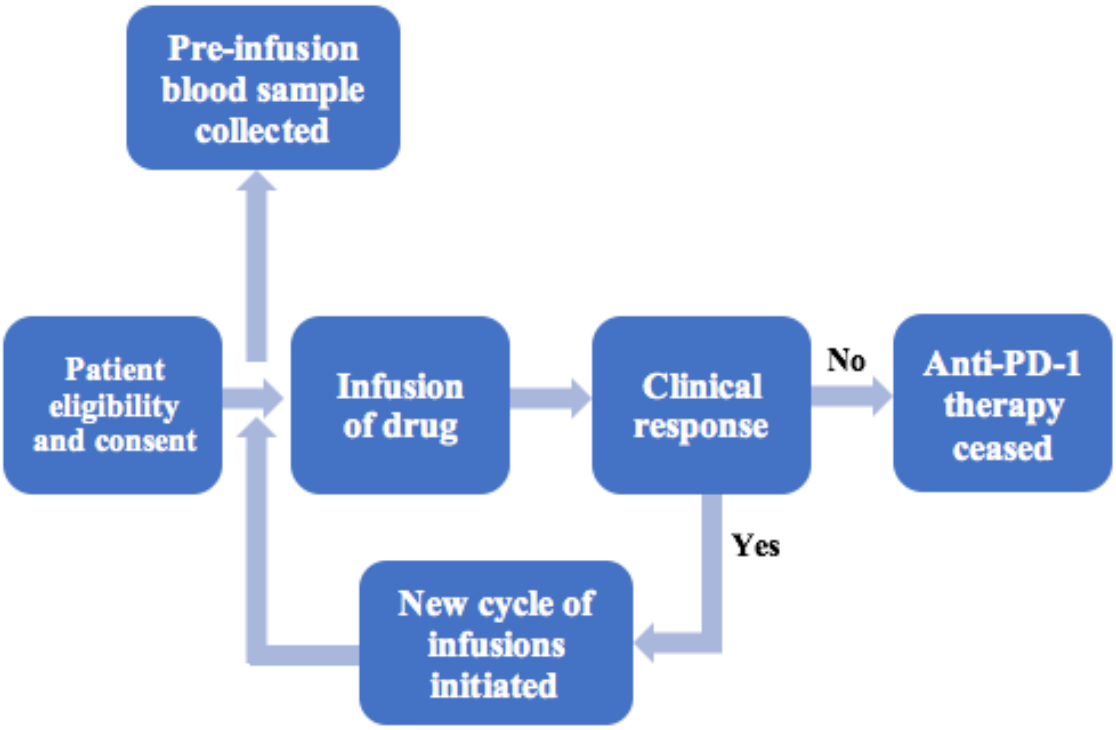

Figure 3.1: Procedure of anti-PD-1 therapy and collection of blood samples in this study. After assessment of patient eligibility and consent, blood samples were collected from patients immediately before infusion of an anti-PD-1 agent (Q3W). At 12 weeks, a CT scan was performed to assess response (according to RECIST criteria). Complete response, partial response or stable disease allowed the patient to initiate a new cycle of anti-PD-1 treatment, whereas progressive disease led to cessation of treatment. No further blood samples were collected from patients who stopped therapy.

3.2.3 Reagents and Equipment

Wash buffer consisted of 0.1% tween in PBS and washing was completed using Wellwash™ microplate washer (Thermo Scientific™). Nivolumab (Opdivo®), Pembrolizumab (Keytruda®), Adalimumab (Humira®) was obtained from the residual contents of vials that had been used for patient infusions. All samples and standards were diluted in 1% milk powder (MP) in PBS. 1% MP was prepared by diluting 5% non-fat dried milk powder (Anchor) (stored at 4°C in PBS and azide) in PBS. Goat Anti-human IgG (Fc specific-F(ab')2) was obtained from Sigma and stored at 500µg/ml with H_2O. Recombinant human TNFα (*E.coli* derived, carrier protein free, R&D systems) was stored at 10µg/ml in PBS. Recombinant human His-tagged PD-1 was obtained from R&D systems, and Streptavidin-HRP (0.83g/L) from Dako. All reagents were stored at 4°C, unless noted otherwise. Optical density was measured using a Multiskan™ GO microplate spectrophotometer (Thermo Scientific™) with SkanIt™ Software version 4.1 (Thermo Scientific™).

3.2.4 Biotin-therapeutic antibody conjugates

Pembrolizumab was biotinylated using EZ-Link NHS-PEG4-Biotinylation Kits (Pierce, 21455), according to manufacturer's instructions. In brief, 170µl of water was added to the NHS-PEG_4-Biotin and transferred to the therapeutic antibody solution, containing 2mg/ml of pembrolizumab in PBS. After incubation (30 minutes, room temperature), non-reacted NHS-PEG_4-Biotin was removed using a desalting column. After dialysis, biotinylated-therapeutic antibody was stored at 4℃. Nivolumab was biotinylated using the same method described above by Dr. Barry Hock (Haematology Research Group, University of Otago, Christchurch, NZ). The specificity and optimal dilution of biotinylated antibodies was analysed using a capture ELISA. Plates were coated with TNF, PD-1, Goat anti-human IgG (positive control) or PBS (negative control) and supplemented with either nivolumab (2µg/ml-0.25µg/ml), pembrolizumab (2µg/ml-0.25µg/ml), adalimumab (1µg/ml) or no drug (1% MP in PBS).

3.2.5 Capture ELISA for Analysis of Drug Concentrations

Enzyme-linked immunosorbent assay (ELISA) for analysis of nivolumab and pembrolizumab concentrations were performed in 96 well microtiter plates (Nunc-Maxisorp) precoated (o/n, 4°C) with 100µL/well PD-1 at 500ng/mL in PBS. Plates were washed (Tween 20/PBS x3) and blocked with 1% milk powder for 30 minutes at 37°C. Following removal of blocker, samples (100µg/well) were added and plates incubated (2 hours, 37°C). Following washing plates were incubated with 100µg/well Goat anti-human IgG biotin (Fc specific-F(ab')2, Sigma) diluted 1 in 1000 in 1% MP supplemented with 2% goat serum. After incubation (1 hour, 37°C) and subsequent washing, wells were incubated (30 minutes, 37℃) with 100µl/well streptavidin-HRP (Sigma) diluted 1 in 7000 in 0.1% bovine serum albumin/PBS. Wells were washed and developed using 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (5 minutes, room temperature). The reaction was stopped by acidification (2.5 mmol H_2S0_4) and optical density was read at 450nm. The key steps of this method are shown in Figure 3.2.

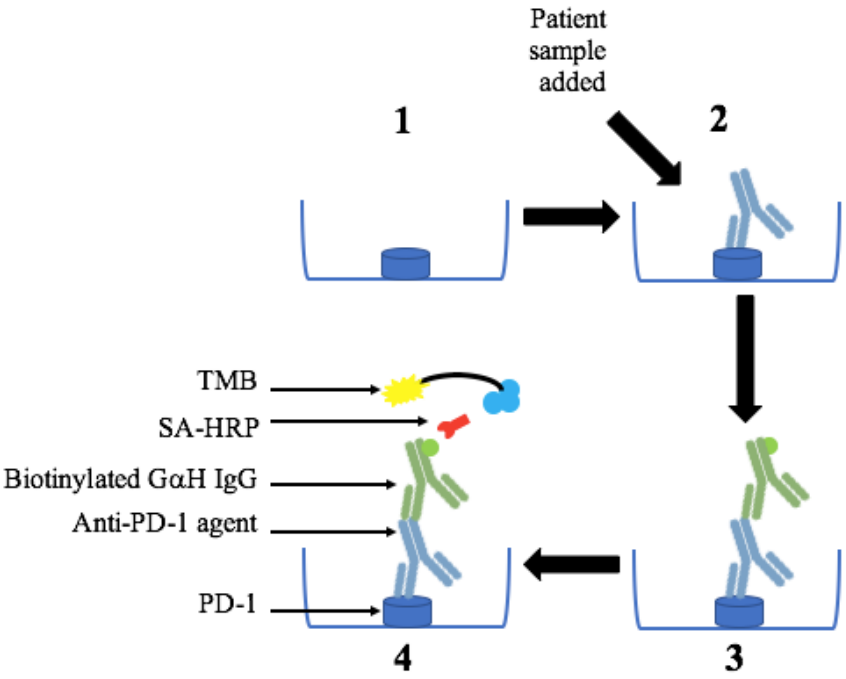

Figure 3.2: Key steps of the capture ELISA method used for analysis of anti-PD-1 drug concentrations. (1) An ELISA plate is coated with PD-1. (2) Patient serum is added to a PD-1 coated ELISA plate and anti-PD-1 therapeutic antibodies (nivolumab or pembrolizumab) bind to PD-1. (3) Biotinylated goat anti-human IgG is added and binds to the anti-PD-1 agent. (4) SA conjugated to HRP binds to biotin and reacts with TMB to form a detectable coloured product.

3.2.6 Calibration Curves

Calibration curves for the estimation of drug concentrations were prepared from known stock concentrations of nivolumab and pembrolizumab by serial dilution in 1% MP plus 2% goat serum. Samples of known concentrations were analysed by capture ELISA following the same method described above (section 3.2.5). Previous experiments conducted by the HRG indicated a range of dilutions suitable for the generation of calibration curves in the current study. Initially, nivolumab and pembrolizumab calibrators were used in the range of 200-0.8ng/ml and 100-0.4ng/ml, respectively. However, the concentration of calibrators was too high and large error bars were produced questioning the accuracy of the curve. To optimize the standard curve, a second capture ELISA was conducted. In the second approach, optimized calibration curves were produced using nivolumab and pembrolizumab calibrators ranging from 50-0.2ng/ml. Serum drug concentrations were interpolated from the optimized calibration curve that was obtained using a 4-parameter logistic curve fitting program (GraphPad Prism version 6.0).

3.2.7 Standard Bridging ELISA for Detection of ADA

A bridging ELISA for detection of anti-drug antibodies was performed in a flat-bottom 96 well microtiter plate (Maxisorp). Key steps of the bridging ELISA method used in this study are shown

in Figure 3.3A. The plate was coated with nivolumab or pembrolizumab at a concentration of 5µg/ml in PBS by adding 100µl per well. Following incubation (2 hours, 37°C), the plate was washed and blocked for 30 minutes (37°C) with 1% milk powder (1% nonfat milk powder [98% fat free, Anchor] in PBS). 100µl of sample, diluted 1 in 50 in 1% MP, was added to the wells and incubated (1.5 hours, 37°C). The plate was washed and 100µl of biotinylated therapeutic antibody (diluted 1 in 1000 in 1% MP supplemented with 1% Goat serum) was added. Following incubation, (1 hour, 37°C), the plate was washed and wells were incubated with 100µl SA-HRP diluted 1:7,000 in 0.1% BSA/PBS before washing and development (5 minutes, room temperature) with TMB substrate. The reaction was stopped by acidification (2.5mmol H_2S0_4) and the optical density was read at 450nm.

3.2.8 ACE assay (Modified Bridging ELISA) for Detection of ADA

ELISA for detection of anti-drug antibodies were performed in flat-bottom 96 well microtiter plates (Maxisorp). Key steps of the ACE ELISA used in this study are shown in Figure 3.3B. Plates were coated with nivolumab or pembrolizumab at a concentration of 5µg/ml in PBS by adding 100µl per well. Following incubation (2 hours, 37°C), plates were washed and incubated for 30 minutes (37°C) in 200µl wash buffer. In fresh 96 well V bottom microtiter plates (Maxisorp), aliquoted samples (15µl) were acidified with 100µl acetic acid (300mM) to dissociate ADA-free drug complexes. Following incubation (40mins, 37°C) 100µl neutralizing buffer (Tris 0.5M, pH 9.5/0.2% Tween 20) was added to the samples. Neutralised samples (100µl) were then transferred to the drug-coated plates and incubated overnight (4°C) to allow ADA the opportunity to bind solid-phase drug. The following day, plates were washed once in 0.1% Tween and twice with water. Bound ADA was then eluted by addition of 65µl 300mM acetic acid for 10 minutes at room temperature. Fresh 96 well microtiter plates were loaded with 50µl of Tris buffer (1M, pH 9.5). 50µl of the acid eluate was transferred to the Tris buffered plates and incubated for 1.5 hours at 37°C to allow binding of eluted ADA to the wells. Following washing (0.1% Tween), plates were blocked with 1% milk powder (1% nonfat milk powder [98% fat free, Anchor] in PBS) for 30 minutes at 37°C. 100µl of biotinylated therapeutic antibody (diluted 1 in 1000 in 1% MP supplemented with 1% Goat serum) was added and incubated (1 hour, 37°C). Plates were washed before SA-HRP incubation and colour development as described above for the detection of ADA using a bridging ELISA. The greater the amount of captured anti-drug antibodies in a sample, the more TMB substrate formed and subsequently, the higher the optical density of the sample. Mean optical density of samples was expressed as a percentage change, relative to the overall mean

optical density measured in all drug naïve (control) patient sera. We classified positive samples, as samples with a significant increase in mean optical density compared to control samples.

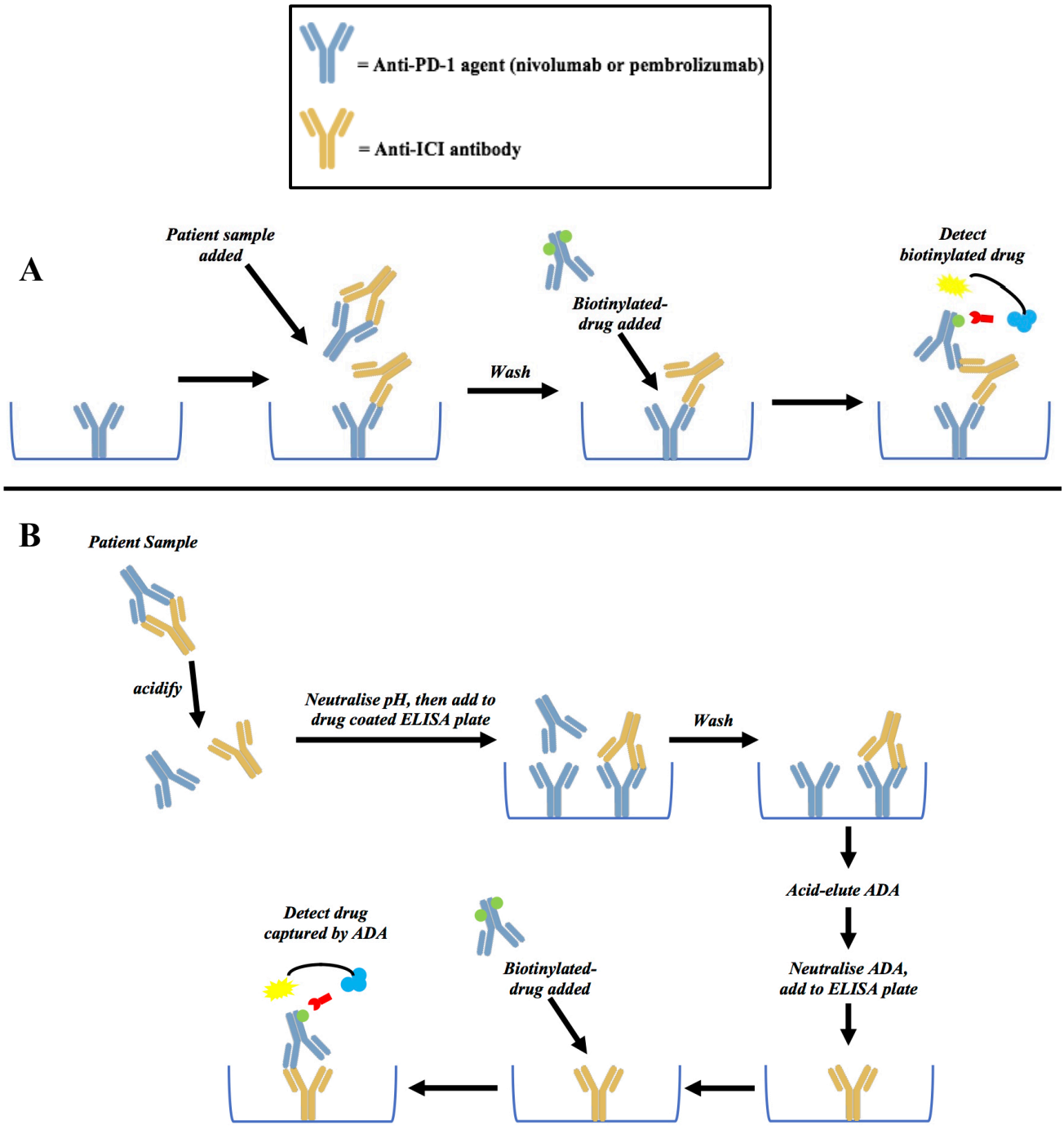

Figure 3.3: Key steps of the Bridging ELISA (A) ACE ELISA (B) used for detection of anti-drug antibodies in this study. (A) ADAs from patient serum bind to anti-PD-1 antibody (drug) coated on an ELISA plate. These ADAs are subsequently detected with anti-PD-1 antibody (drug) labelled with biotin. Biotinylation allows for detection of the anti-PD-1 antibody and attached ADA with an enzyme and measurable substrate. (B) Firstly, acidification of patient serum dissociates ADA-drug complexes into free anti-PD-1 antibody and free ADA. Following neutralization, ADAs are affinity captured in the in the presence of solid-phase drug. After the removal of free drug by washing, acid-eluted ADA is transferred onto a fresh ELISA plate. Captured ADAs can then be detected through the addition of labelled therapeutic antibody attached to an enzyme and substrate.

3.2.9 Statistical analysis

All analyses were carried out using Microsoft Excel version 15.38 and GraphPad Prism version 6.0 (San Diego, CA, USA). Values were expressed as mean ± standard error of the mean.

3.3 Results

3.3.1 Patient Population

A total of 23 trough serum samples from 8 patients undergoing immune checkpoint inhibitor therapy were analysed in this study. Of these patients, 6 (75%) were treated with pembrolizumab and 2 (25%) with nivolumab monotherapy. 16 samples were obtained from pembrolizumab-treated patients, whilst the remaining 7 samples were collected from patients receiving nivolumab. Trough samples from patients varied in start time collection point, ranging from prior to infusion 1 to prior to infusion 24. The maximum number of consecutive samples collected from one patient throughout treatment was four. Characteristics of patients included in analysis, undergoing ICI-therapy, are shown in Table 3.1. An additional 23 samples from drug-naïve diagnostic laboratory samples were analysed as negative controls.

Table 3.1: **Clinical Characteristics and responses of ICI-treated patients analysed in this study**

Patient	Anti-PD-1 agent	Age	Gender	Best Overall Response	irAEs	No. of samples provided	Cycle of first sample collection
1	Pembrolizumab	59	m	PR	Cough, fatigue, dyspnea, arthralgia, pneumonitis, hypersensitivity reaction, rash	4	3
2	Nivolumab	63	f	PR	Cough, fatigue, dyspnea, diarrhea, hypoadrenalism, decreased appetite	2	21
3	Pembrolizumab	68	m	PR	Rash, constipation, diarrhea, hypothyroidism, fatigue	4	8
4	Nivolumab	77	f	PR	Fatigue, lymphopenia	5	24
5[a]	Pembrolizumab	-	f	-	-	3	11
6	Pembrolizumab	56	f	PR	Nausea, fatigue	3	9
7	Pembrolizumab	57	f	Not yet evaluated	Hypersensitivity reaction	1	3
8[b]	Pembrolizumab	-	m	-	-	1	1

[a,b] Missing data

3.3.2 Analysis of reagent specificity

Prior to analysis of patient serum samples, assays were performed to confirm the specificity of reagents utilized in the drug and ADA ELISAs. Biotinylated anti-PD-1 antibodies had been prepared in the laboratory as described in section 3.2.4 and were analysed to confirm their specificity and the optimal dilution for their use. Biotinylated nivolumab and pembrolizumab strongly bound PD-1 but did not react with a control recombinant protein, TNF. In contrast, the anti-TNF antibody adalimumab did not react with PD-1 but strongly bound TNF. Results of this capture ELISA are displayed in Figure 3.4.

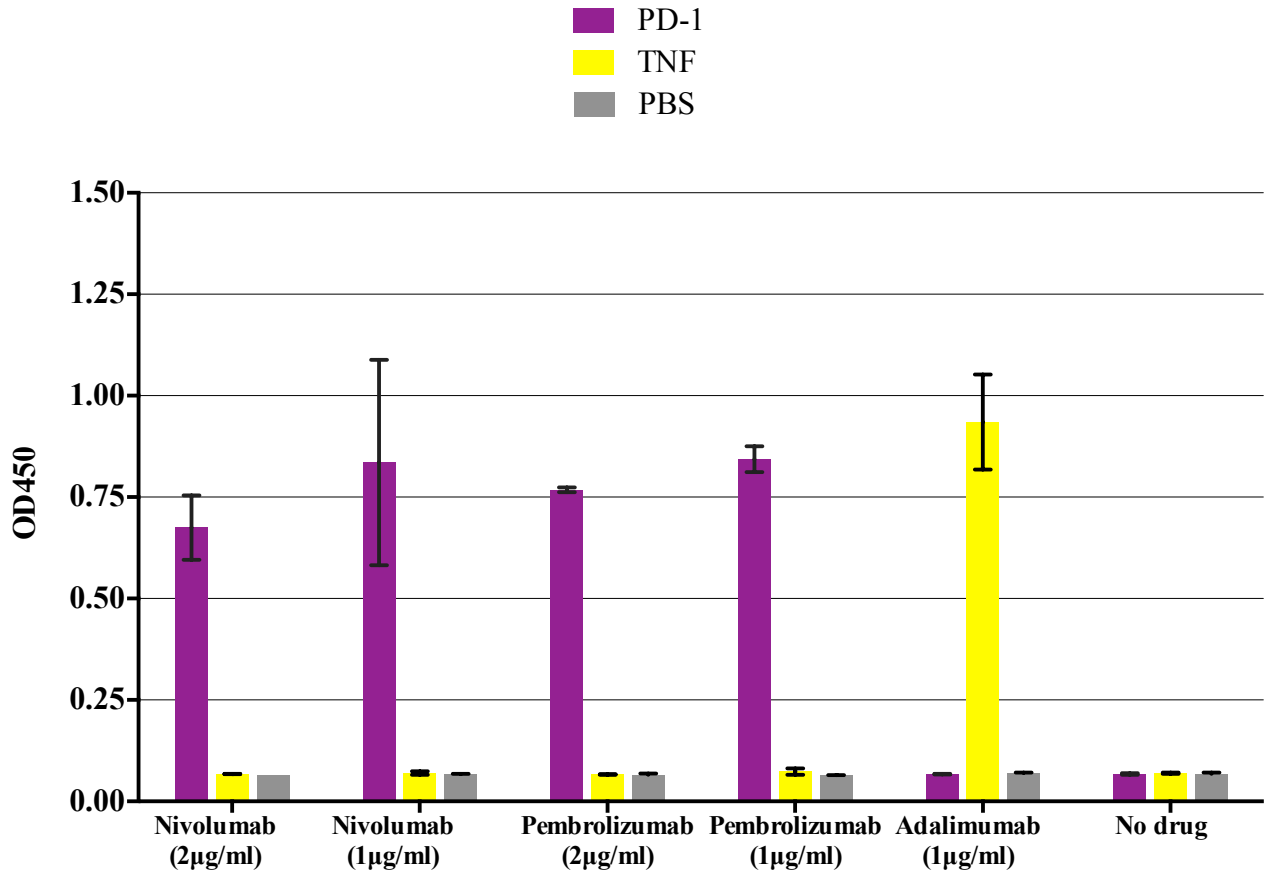

Figure 3.4: Binding specificity of nivolumab and pembrolizumab. The specificity and optimal dilution of biotinylated anti-PD-1 antibodies was analysed using a capture ELISA. Plates were coated with TNF (positive control), PD-1 or PBS (negative control) and supplemented with either nivolumab, pembrolizumab, adalimumab or no drug. Biotinylated anti-PD-1 agents bound PD-1 and adalimumab bound TNF. Data is expressed as mean ± SEM.

3.3.3 Analysis of Patient Drug Concentrations

Following optimization of the calibration curve (Figure 3.5 and Figure 3.6), a capture ELISA was used to analyse drug concentrations in patient samples. Initially, investigation of drug concentrations was completed in a subset of patient samples, specifically in three patients treated with pembrolizumab (patient 1, 5 and 6) and two patients treated with nivolumab (patient 2 and 4), to determine the appropriate dilution of patient samples to quantify drug levels. At first, patient samples

were diluted 1 in 1000 in 1% MP. However, mean optical densities from 4 of the 5 patients analysed were outside the range of the standard curve and thus, the drug concentration in these samples was not able to be quantified. As the patient samples were above the range of the standard curve, a subsequent ELISA was completed on samples from patients 2,4,5 and 6 ranging in dilutions from 1 in 1000 to 1 in 16,000. Drug concentrations from samples diluted 1 in 8000 and 1 in 16,000 were able to be interpolated from the calibration curves. However, a 1 in 8000 dilution was deemed a suitable dilution for subsequent analysis of drug concentration in patient samples. Values from samples of this dilution fell within the linear part of the calibration curve permitting greater accuracy of drug concentrations in further analysis.

Due to time constraints and a low number of nivolumab-treated patients available for analysis, no further analysis was completed on samples from patients 2 and 4. Instead, a capture ELISA was used to analyse drug concentrations of all samples belonging to the 6 pembrolizumab-treated patients. The results of this ELISA are shown in Figure 3.7 and Figure 3.8. The mean trough concentration of pembrolizumab in this patient subset was 17.6μg/ml, ranging from 0.79 to 86.7μg/ml. Patient 1 had the lowest levels of pembrolizumab and was the only patient to display concentrations lower than 1μg/ml. Specifically, patient 1 had pembrolizumab levels of 0.83, 0.79, 0.80 and 1.07μg/ml prior to treatment cycles 3, 4, 5 and 6, respectively. Patient 8 had a similarly low level of pembrolizumab (1.47μg/ml). The maximum trough level of pembrolizumab was observed in patient 5 who had a concentration of 86.7μg/ml prior to cycle 11 of treatment. The concentration of pembrolizumab in patient 5 decreased to 24.7 and 20.6μg/ml in subsequent treatment cycles. In contrast, the trough concentration of pembrolizumab in patient 6 increased throughout treatment from 14.8μg/ml prior to cycle 9 to 30.7μg/ml prior to cycle 10. The same trend occurred in patient 3, who exhibited slightly increased levels throughout treatment from 15.9μg/ml prior to cycle 8 and 9 to 18.07 and 18μg/ml prior to cycle 10 and 11, respectively. The only available sample from patient 7 had a level of 12.12μg/ml of pembrolizumab. Analysis of samples from drug naïve (control) patients generated low levels of background signal, equaling pembrolizumab concentrations of less than 1.5μg/ml.

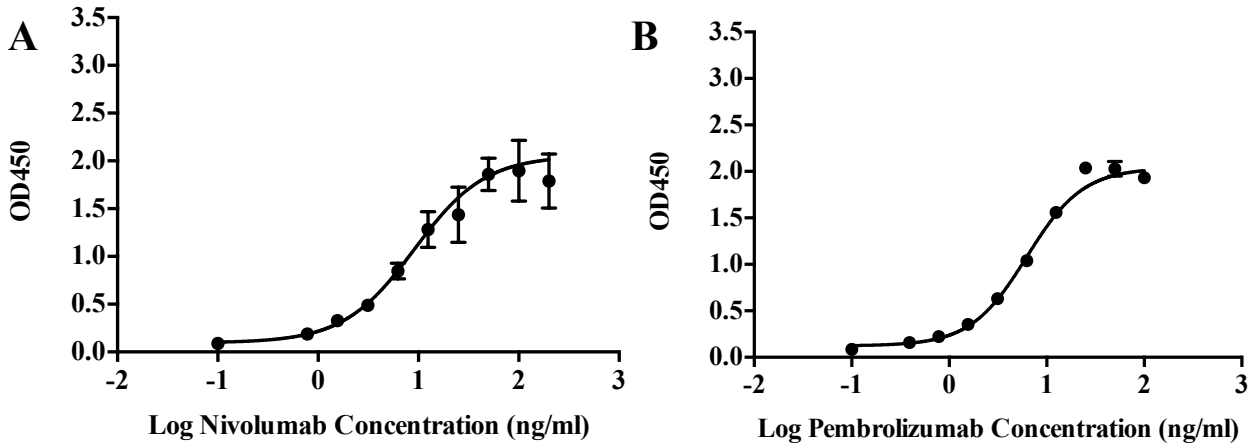

Figure 3.5: Initial calibration curves of anti-PD-1 agents. Calibration curves were prepared with a capture ELISA using nivolumab (A) and pembrolizumab (B) calibrators ranging from 200-0.8ng/ml and 100-0.4ng/ml, respectively. Data is represented as mean ± SEM.

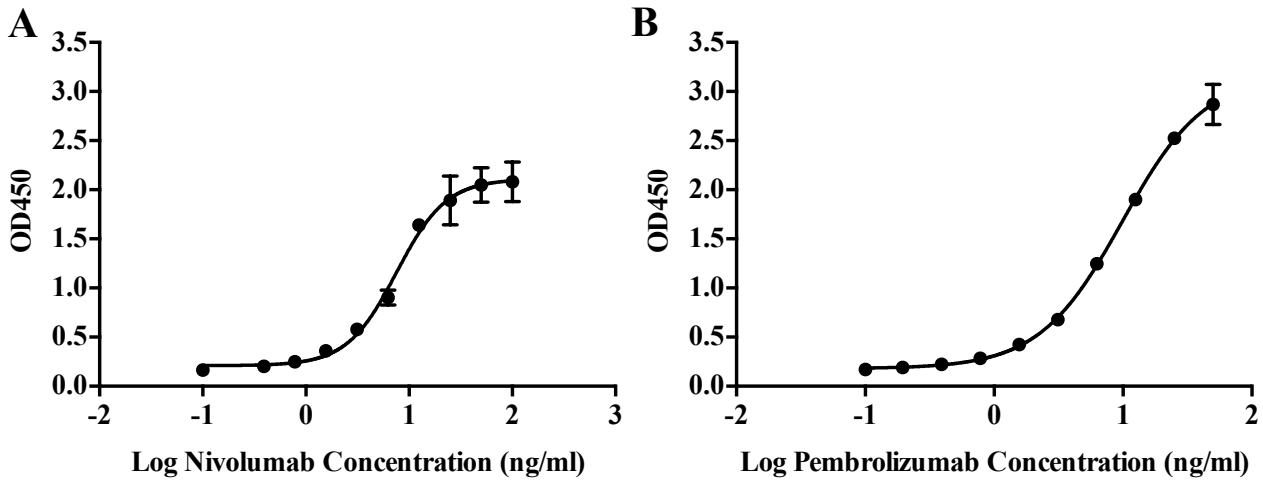

Figure 3.6: Optimised calibration curves of anti-PD-1 agents. Calibration curves for analysis of drug concentrations were prepared with a capture ELISA using nivolumab (A) and pembrolizumab (B) calibrators ranging from 50-0.2ng/ml. Data is represented as mean ± SEM.

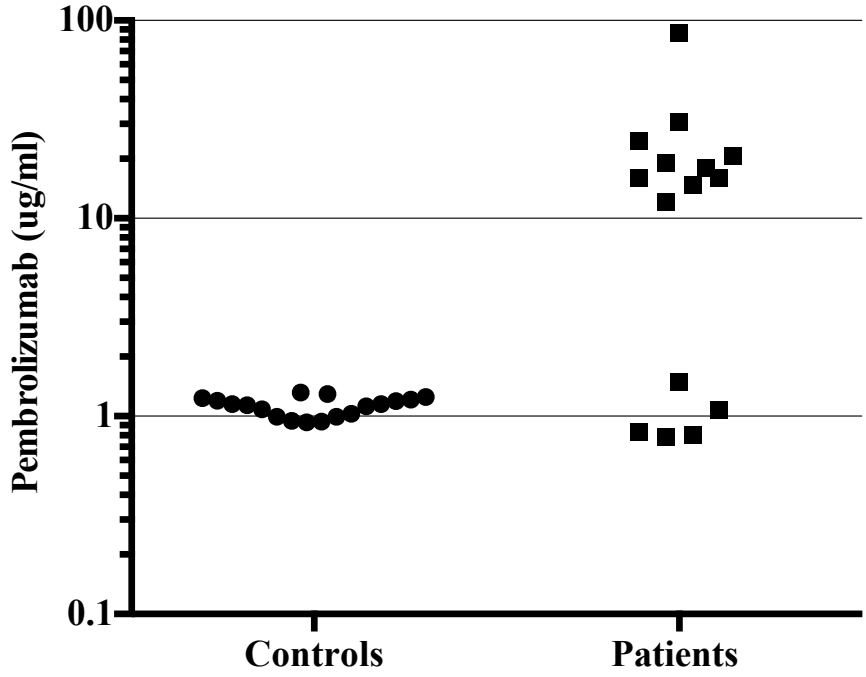

Figure 3.7: Circulating pembrolizumab concentration in patients. The concentration of drug in samples from pembrolizumab-treated patients (n=6) and drug naïve individuals (n=18) were analysed using a capture ELISA. Resulting optical densities were used to determine pembrolizumab concentration from the calibration curve. Markers represent the mean pembrolizumab concentration of individual samples from each patient analysed in duplicate.

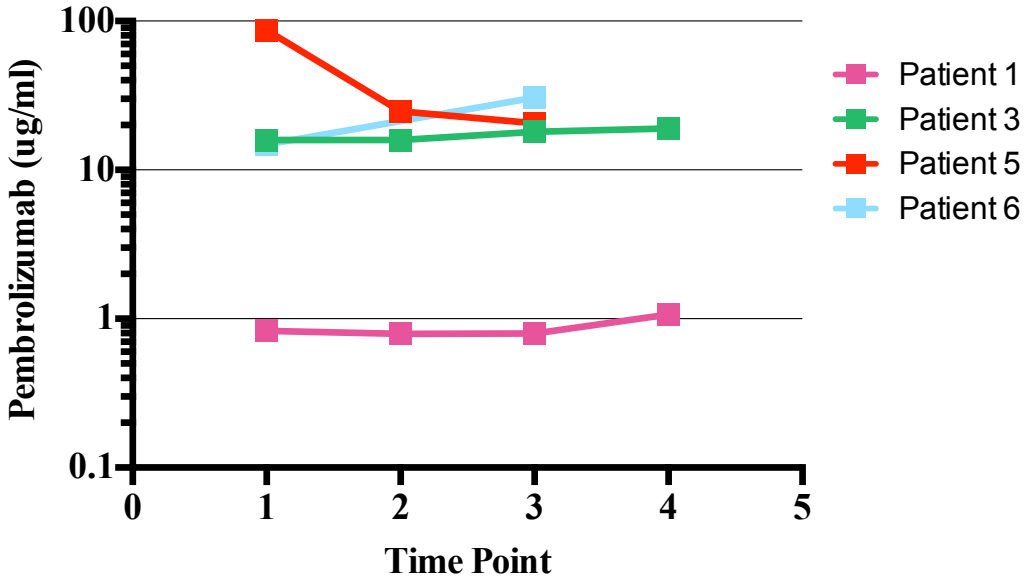

Figure 3.8: Circulating concentration of pembrolizumab in patients throughout treatment. A capture ELISA was used to determine the concentration of pembrolizumab in four patients who provided multiple samples throughout treatment. Time point 1 corresponds to the time at which the patient provided their first sample, before collection of their second (time point 2), third (time point 3) and fourth (time point 4) samples at three-weekly intervals. Markers represent the mean pembrolizumab concentration of individual samples analysed in duplicate.

3.3.4 Detection of ADAs in Patient Serum using an ACE ELISA

The in-house developed ACE assay (modified bridging ELISA) was used to measure levels of anti-pembrolizumab antibodies in 6 patients undergoing pembrolizumab monotherapy. The results of this assay are displayed in Figure 3.9. Four samples displayed a higher mean optical density than all samples tested. These four samples, belonging to patient 1, had a 90, 81, 63 and 43% increase in optical density compared to control samples. Samples belonging to the remaining 5 pembrolizumab-treated patients ranged in percentage change of mean optical density from -4% to 18%.

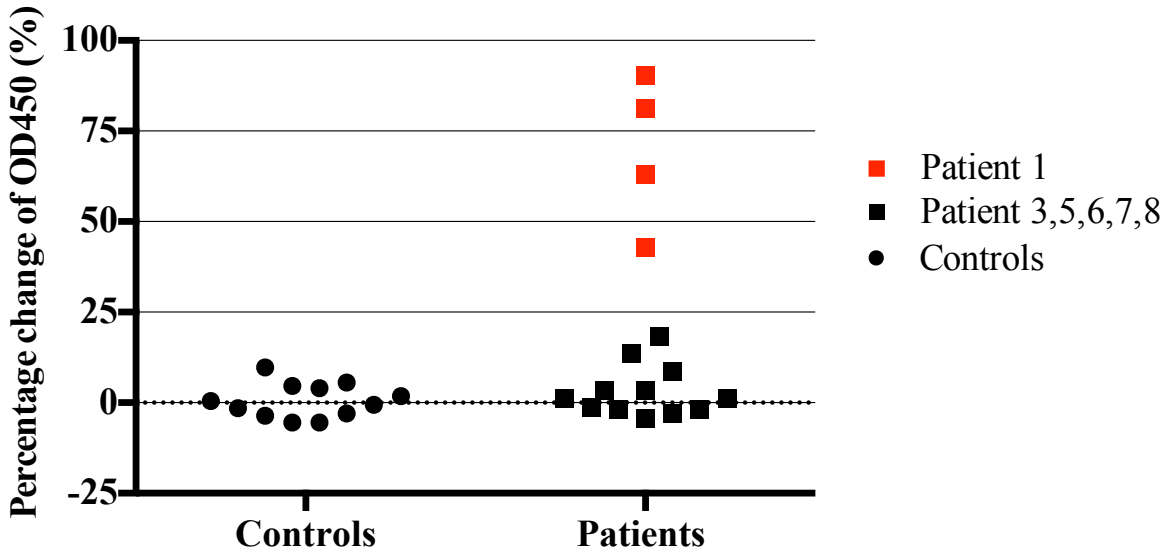

Figure 3.9: Anti-drug antibody levels in patient samples. An ACE-ELISA was used to detect ADAs in 16 samples from 6 pembrolizumab-treated patients. ADAs were detected in four samples from one patient (patient 1). The mean optical density of samples was expressed as a percentage change, relative to the overall mean optical density measured in all drug naïve (control) patient sera.

3.3.5 Analysis of ADA in Patient Serum using a Standard Bridging ELISA

To investigate concordance between ACE and bridging ELISA-based methods, sera from patients previously analysed using the ACE ELISA were subjected to analysis with a standard bridging ELISA method, as described in section 3.2.7. Serum from a patient positive for ADAs using the ACE assay (patient 1) and from a patient negative for ADAs using the ACE assay (patient 5) were analysed using the bridging ELISA. Although patient 1 and patient 5 were pembrolizumab-treated, a section of wells was coated with nivolumab, rather than pembrolizumab to act as a negative control. Further controls included the analysis of samples containing serum from two drug-naïve patients (negative control), goat serum (negative control) or goat anti-human IgG (positive control).

Results of analysis using the bridging ELISA are shown in Figure 3.10. No anti-drug antibodies were detected in patient 1 or patient 5. The mean optical densities of pembrolizumab-coated wells supplemented with serum from patient 1 and patient 5 were 0.091 and 0.082,

respectively. Similarly, pembrolizumab-coated wells of drug-naïve patient serum displayed mean optical densities of 0.082 (control patient A) and 0.084 (control patient B). The mean optical density of these samples in nivolumab-coated wells was 0.073 (patient 1), 0.073 (patient 5), 0.072 (control patient A) and 0.073 (control patient B). No antibodies were detected in the sample containing goat serum, showing mean optical densities of 0.084 in pembrolizumab-coated wells and 0.073 in nivolumab-coated wells. In contrast, antibodies were detected in the sample containing goat-anti human IgG. In pembrolizumab and nivolumab-coated wells containing goat-anti human IgG, the mean optical density was 1.01 and 0.76, respectively.

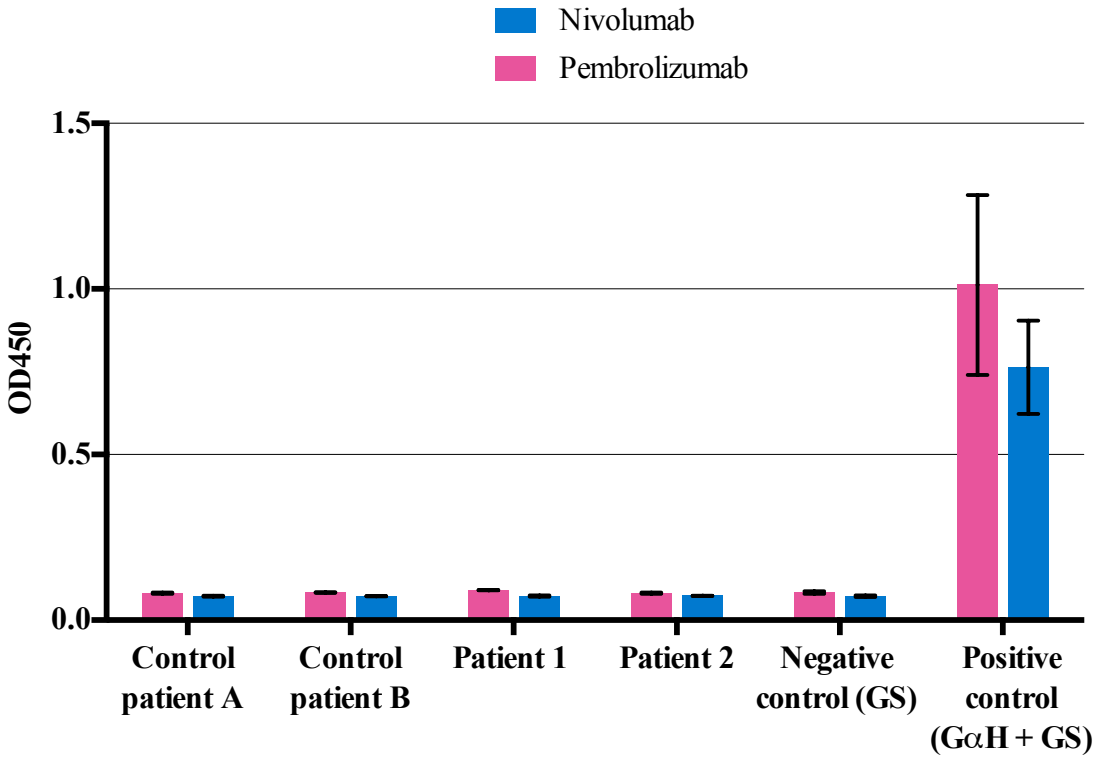

Figure 3.10: Levels of ADAs in samples analysed with bridging ELISA. Selected patient samples and controls were analysed for ADAs using a standard bridging ELISA. No ADAs were detected in patient samples. GS, Goat serum; GαH, Goat anti-human antibody. Data is represented as mean ± SEM.

3.3.6 Relationship between ADA, Drug Levels and Patient Response

The prevalence of anti-pembrolizumab antibodies (ADAs) in patient 1 was accompanied by low trough levels of pembrolizumab compared to other patients, as shown in Figure 3.11. Four consecutive trough samples, commencing prior to infusion number 3, were available for analysis from patient 1. According to a reduction in mean optical density, the level of ADAs in patient 1 decreased throughout treatment (Figure 3.11). The mean optical density reduced from 1.16 prior to infusion three to 0.154 prior to infusion 6. The mean optical densities from trough samples taken before infusion four (0.483) and infusion five (0.233) also showed a decrease in anti-

pembrolizumab antibodies. Although the presence of anti-pembrolizumab antibodies was associated with lower drug levels in this patient (range 0.79-1.07µg/ml), the reduction of ADAs throughout treatment was not associated with a significant increase in pembrolizumab trough levels, which stayed relatively constant throughout treatment, only increasing by 0.24µg/ml (Figure 3.11). At the time of analysis, patient 1 had exhibited a partial response to treatment as indicated by an initial restaging CT scan and according to RECIST criteria. However, throughout treatment, patient 1 experienced a range of toxicities including fatigue, cough, dyspnea, arthralgia, pneumonitis and rash. A hypersensitivity reaction was also reported for this patient during their third infusion of pembrolizumab.

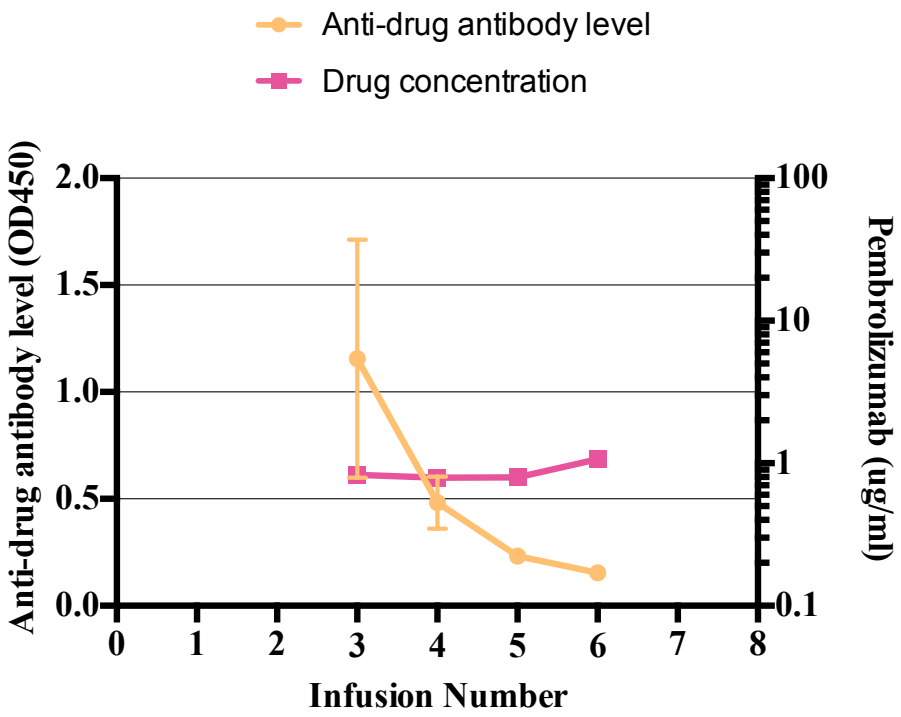

Figure 3.11: Drug concentrations and anti-drug antibody levels in Patient 1 throughout treatment. Four samples from patient 1, collected at infusion 3 and three subsequent infusions, were analysed using a capture ELISA and ACE-ELISA for circulating pembrolizumab concentrations and ADAs, respectively. Anti-drug antibody levels are represented as mean ± SEM and drug concentration markers represent the mean pembrolizumab concentration of individual samples analysed in duplicate.

3.4 Discussion

Equivalent to other therapeutic antibodies, the administration of ICI antibodies has the potential to elicit undesirable immunogenicity, leading to downstream consequences on patient outcome.[136,137,158] Of particular relevance, are clinical reports of decreased efficacy and/or increased toxicity occurring within patients treated with mAbs.[140,142,144,152] Increasing evidence suggests monitoring of therapeutic antibody levels and their respective ADAs can help guide treatment

decisions and improve the clinical outcomes of patients.[173, 174] However, current detection methods possess a range of limitations, restricting their routine use in general practice.[146] Thus, this chapter incorporated an in-house developed ELISA in the measurement of drug and anti-drug antibody levels of patients undergoing ICI-therapy and analysed their association with patient outcome.

Initially, this chapter explored circulating drug concentrations of patients treated with anti-PD-1 agents. The mean pembrolizumab trough concentration of samples from patients in our study (17.6μg/ml) was slightly lower than in previous studies, which have estimated a mean trough concentration of 23.5μg/ml.[148] An explanation for this slightly lower mean concentration may relate to the proportion of samples in this study belonging to patient 1 (4 out of 16, 25%). All four samples from patient 1 had low pembrolizumab concentrations of less than 1.5μg/ml and coincided with the detection of anti-drug antibodies, which is to be discussed in subsequent paragraphs. Besides patient 1, patient 5 (86.7μg/ml) and patient 8 (1.47μg/ml) also had pembrolizumab concentrations outside the range expected from published data.[148,175] These outliers may be due to flaws in experimental technique or inter-individual variations in pharmacokinetic parameters, resulting in different circulating drug concentration-time profiles after administration of similar doses to different patients.[175]

The cycle at which samples were collected from patients may be a contributing factor to the wide range of pembrolizumab-concentrations observed in this study (0.79-86.7μg/ml). Although, all serum samples from pembrolizumab-patients were collected at trough level, the samples were collected from patients during different cycles of treatment (as they came through the clinic during this study). With repeated administration, the concentration of drug is expected to gradually increase until it reaches a steady state, as shown in Figure 3.12.[175] Thus, patients further along in their treatment regime are expected to have higher drug levels than patients at the beginning of treatment. This is exemplified in this study by patients 5 and 6, who not only had the highest circulating concentration of pembrolizumab, but also had received the greatest number of pembrolizumab infusions prior to the collection of blood samples.

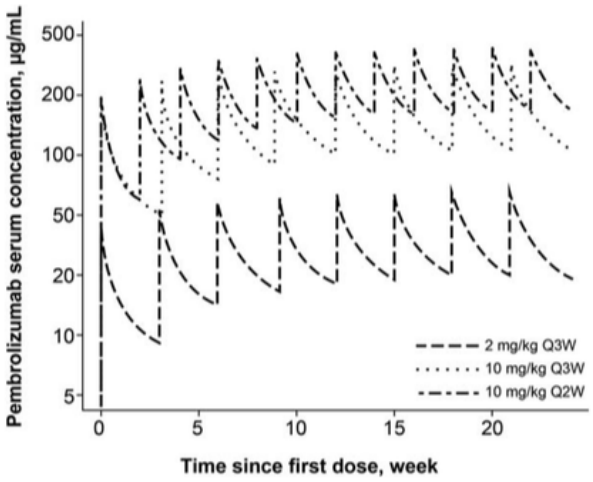

Figure 3.12: Predicted pembrolizumab concentration-time profiles during multiple dosing with the regimens included in clinical trials for melanoma and NSCLC. Reprinted from Ahamadi et al (2017).[175]

Previous immunogenicity analyses have estimated a low prevalence of anti-drug antibodies in patients undergoing ICI-therapy.[148,182] In an assessment report, conducted by the European medicines agency, anti-drug antibodies were detected in 1.1% (29 out of 2,632) of patients treated with pembrolizumab.[148] In contrast, our study indicated a higher prevalence of anti-drug antibodies in pembrolizumab-treated patients. Of the 6 pembrolizumab-treated patients analysed in our study, ADAs were detected in 1 patient (17%). There are several possible explanations for the higher prevalence observed in our study. Obviously, caution needs to be exercised when interpreting data from a small explorative study such as this and one explanation is that our data may be due to chance. However, it is interesting to note that when analysis was completed using a conventional bridging ELISA, no anti-drug antibodies were detected in any of the 6 pembrolizumab-treated patients. Therefore, a probable explanation for contrasting results, not only within our study but with previous literature, is the use of different methods to detect ADAs. A key problem with bridging ELISAs is the inability to detect ADAs in the presence of drug and monovalent IgG4 ADAs, limitations we sought to overcome in this study through the use of a modified ACE-ELISA.[146,158] As with many other immunogenicity studies, the assessment report described above, analysed samples using a standard bridging ELISA. In our study, ADAs were analysed using both a bridging ELISA and modified ACE ELISA, but were only detected when using the latter method. Our data suggests that bridging ELISAs can produce false-negative results and may therefore, inaccurately estimate the prevalence of anti-drug antibodies in patients treated with pembrolizumab. More importantly, the extent to which anti-drug antibodies negatively influence patient outcome may be under-estimated.

To confirm the patient positive for ADAs (patient 1) in our study was a true positive, samples from this patient could be subjected to further analysis using techniques with known high sensitivity and specificity. For example, the homogenous mobility shift assay, which displays higher sensitivity and specificity than ELISA methods, could be applied. An intended future purpose of the current work is the translation of methodology into a clinical setting. The HMSA requires specialist equipment and laboratory personnel, restricting its use in routine mAb monitoring and thus, was not initially applied to samples in this study.[146]

Based on previous literature regarding therapeutic monoclonal antibodies, we hypothesized that patients who produce anti-drug antibodies exhibit (1) a decreased efficacy of treatment and/or (2) an increased risk of toxicity. We also hypothesized (3) that these patients would have lower levels of circulating drug. Part 1 of our hypothesis was not supported by results of the current study. The sole patient who was identified as producing anti-pembrolizumab antibodies (patient 1), exhibited a partial response to treatment. This response may have been explained by the presence of an effective concentration of pembrolizumab circulating within the patient. Surprisingly, however, all four samples belonging to patient 1 contained low drug concentrations, conflicting our third hypothesis, and patient 1 was the only patient analysed to have drug levels less than 1μg/ml. A possible explanation for the partial response of patient 1 to treatment is that a minimum effective concentration of pembrolizumab was present prior to collection of trough blood samples. In the days following infusion of pembrolizumab, drug levels may have been high enough to induce a therapeutic effect and produce a partial response in patient 1.

However, analysis of drug and anti-drug antibody levels is not usually performed at peak time points due to the inability of ELISAs to accurately detect anti-drug antibodies in the presence of high levels of drug.[146] As to why low drug levels were detected in trough samples remains to be determined, but may be attributed to the ability of anti-drug antibodies to increase clearance of mAbs. The binding of anti-pembrolizumab antibodies to pembrolizumab to form large immune complex may trigger endogenous elimination processes, promoting clearance of the drug.[158] The subsequent clearance of the drug (complexed with ADA) may explain the low drug levels present in patient 1 and their inability to accumulate higher levels of drug even with repeated infusions. This is supported by a small increase of pembrolizumab levels in patient 1 throughout treatment, in conjunction with declining levels of ADAs. However, the small increase observed throughout treatment (0.24μg/ml) is unlikely to be of clinical relevance. It would be interesting to examine the levels of drug and anti-drug antibodies of patient 1 in subsequent treatment cycles to examine whether drug levels of this patient continued increasing to clinically relevant levels. It should be

noted that serum samples analysed from patient 1 were collected from infusions 3. Although these infusion time points corresponded with a partial response to treatment, this patient may have had clinically relevant levels of pembrolizumab high enough to produce a response, prior to collection of their first sample. This is not outside the realms of possibility as exemplified by patient 5, who experienced a significant decrease in pembrolizumab levels throughout treatment.

An alternative explanation for the response to treatment observed in patient 1, is the speculation that anti-drug antibodies could elicit immune responses that are beneficial during ICI-therapy. In contrast to extensively studied mAbs used in autoimmune disease, the intention of ICI antibodies is to stimulate an immune response rather than suppress it.[1,152] Specifically, anti-PD-1 agents primarily target T cells, enhancing their activation.[1] Anti-drug antibodies have previously been reported to have a direct influence on T cells. The formation of immune complexes has been shown to bind Fc receptors on T cells, resulting in their co-stimulation.[176] Additionally, the production of ADAs by B cells utilizes T cell dependent (and independent) pathways.[158] Therefore, the immune response and activation of T cells caused by foreign sequences within anti-PD-1 agents, may be beneficial in forming a downstream response towards tumour cells. However, this idea is not supported by firm evidence and future work is required to uncover the underlying physiological mechanisms of ADAs within patients treated with ICIs before a causal relationship can be determined. Additionally, the majority of samples available for analysis in our study were from patients who experienced a partial response as their best overall response to treatment. To better characterize the negative influence of anti-drug antibodies on treatment efficacy and patient response, future work must involve the analysis of samples from patients who experienced disease progression.

Conversely, part 2 of our hypothesis (i.e. patients who produce anti-drug antibodies exhibit an increased risk of toxicity) was supported in the present study. Patient 1 presented with a wide range of toxicities during ICI-therapy. Of particular note, is the onset of a hypersensitivity reaction. Infusion-related reactions have previously been linked to the production of anti-drug antibodies and may explain the hypersensitivity reaction observed in patient 1.[140] These responses have been attributed to an inflammatory response towards the therapeutic mAb and therefore, an investigation of inflammatory markers in patients who produce ADAs is warranted. Outside the time constraints of this project, we aim to use a Proteome Profiler Human XL cytokine array kit (R&D systems) to measure relative levels of selected human cytokines and chemokines of patient 1. These markers could potentially predict the formation or downstream effects of anti-drug antibodies and immune

complexes, acting as a biomarker throughout treatment. Ultimately, the discovery and use of these markers could lead to improved clinical management and outcomes of patients treated with ICIs. It should be noted, however, that previous studies found not all patients who produce ADAs exhibit decreased efficacy and/or increased toxicity.[140,144,145] Our study describes one patient case of anti-drug antibody production and despite the associations described above, no conclusions can be drawn. Further investigations in a larger cohort of patients is required to determine the role of anti-drug antibodies during ICI-therapy and their potential influence on patient outcome. In addition to a larger sample size, the collection of samples consistently throughout treatment would enable a better understanding of changes in drug and anti-drug antibody levels and their association with clinical events in individual patients.

A drawback of this study is the inability of the in-house developed ELISA to detect anti-drug antibodies in high concentrations of drug. The analysis of samples collected at trough time point, when drug levels are at their minimum, reduces drug interference but re-association of anti-drug antibodies with free drug, rather than solid-phase drug on the ELISA plate, still remains a potential problem. Another potential limitation is the inability to detect non-neutralising anti-drug antibodies. Despite these drawbacks, the ACE-ELISA described in this study displays superior qualities over currently available assays, particularly in the context of routine therapeutic drug monitoring. It is a relatively simple and inexpensive method that can be performed using widely available reagents and laboratory equipment. Additionally, in contrast to alternative ELISA-based methods, the ACE-ELISA allows detection of inhibitory ADAs complexed to drug. To our knowledge, no studies have yet been published incorporating the use of an acid-dissociation ELISA to detect anti-drug antibodies within patients undergoing ICI-therapy.

In the present pilot study, an in-house developed ELISA was used to investigate drug and anti-drug antibody levels in patients undergoing immune checkpoint inhibitor therapy. The work described in this chapter demonstrates that commonly used bridging ELISAs may inaccurately detect the production of anti-ICI antibodies. Importantly, inaccuracies in detection may under-estimate the possible influence of ADAs on patient outcome. Our study describes the case of a patient who produced anti-pembrolizumab antibodies but, displayed both positive (partial response) and negative (toxicity) outcomes. However, due to a small study population, no conclusions can be drawn from the current study regarding the true prevalence of anti-ICI levels or their association with patient outcome. Whilst, the role of anti-drug antibodies is still yet to be determined, the preliminary data obtained from this study warrants further investigation. Future work investigating these parameters in a larger cohort is required to determine the prevalence and role of anti-drug

antibodies during ICI-therapy. Ultimately, future work could lead to the introduction of routine monitoring of ICIs and anti-ICI antibodies in general practice, with these parameters acting as biomarkers throughout treatment.

Chapter 4

4 Discussion

4.1 Summary of findings

The recent success of immune checkpoint inhibitors has garnered attraction worldwide and led to a growing interest into research of these agents. However, the reasons why a subset of patients don't respond to treatment or experience toxicity are poorly understood, a knowledge gap we sought to address in the current study. To prevent a proportion of patients exposed to an ineffective and potentially harmful therapy, this exploratory study aimed to investigate biomarkers that predict patient outcome during anti-PD-1 therapy. In this study, no clinicopathological markers were found to predict patient response, patient survival or the development of toxicity. In contrast to our hypothesis, patients at an increased risk of developing autoimmune diseases did not exhibit an increased risk in developing irAEs during ICI-therapy. However, our results demonstrated that patients typically excluded from clinical trials of anti-PD-1 agents could be effectively treated with immune checkpoint inhibitors, but perhaps carry a greater risk of toxicity.

Furthermore, no immunogenic biomarkers were found to reliably predict outcome, due to the detection of anti-drug antibodies in one sole patient case. In agreement with our hypothesis, the patient who developed anti-drug antibodies experienced toxicities and had a lower level of circulating drug. In contrast, however, a partial response to treatment was observed in this patient, prompting the question as to whether ADAs influence the efficacy of ICI-therapy. Interestingly, we found contrasting results in detection of anti-ICI antibodies between a conventional bridging ELISA and an in-house developed acid-dissociation ELISA, suggesting bridging ELISAs may inaccurately detect the production of ADAs in patients treated with anti-PD-1 agents. Overall, the findings of this exploratory study add to current literature with regards to the use of anti-PD-1 agents in real-life metastatic melanoma patients, but generate further questions surrounding the prevalence and role of anti-drug antibodies during ICI-therapy.

4.2 Future directions

The results in this study must be interpreted in the context of a small study population. Regardless, this study was intended as a preliminary exploratory analysis to identify possible biomarkers of patient outcome to ICI-therapy which, could then be validated with future work. Further research, recruiting patients from multiple locations, over a longer period of time, would enable acquisition of

a larger study population and the ability of significant conclusions to be drawn. Nonetheless, recent published studies investigating immune checkpoint inhibitors have employed smaller sample sizes than the current study and provided fundamental data, in which future work can build on. Likewise, this project provides preliminary data that warrants further investigation in a larger cohort and suggests several directions for future research.

Firstly, outside the constraints of this project and in the immediate future, an investigation into inflammatory markers and patient outcome will be completed. An antibody array will be used to measure inflammatory markers from a patient of interest identified in this study. This patient of interest was one of the 32 patients reviewed in chapter 2 and was subsequently analysed for anti-drug antibodies in chapter 3. Our data suggested this patient is of particular interest due to the production of anti-drug antibodies and the wide range of toxicities they developed throughout treatment. If the production and level of anti-drug antibodies does not directly predict patient outcome, downstream effectors might act as an indication of ADA formation and subsequently, function as a predictor of patient outcome. Based on the central role of ADA-drug immune complexes in the formation of toxicity and their ability to initiate downstream inflammatory pathways, a measurement of inflammatory cytokines and chemokines in this patient will help us, not only characterize the mechanisms of ADAs in patients undergoing ICI-therapy, but also shed light on the underlying physiological pathways mediating irAEs. It is hypothesized that the inflammatory profile of this particular individual will differ from, not only healthy individuals, but also from patients undergoing ICI-therapy who didn't produce ADAs or experience similar toxicological consequences. Depending on the results of this array, individual inflammatory markers could then be pursued further to analyse their association with patient outcome. Samples from a larger group of patients could be subjected to analysis with an ELISA to identify potential biomarkers of patient response, survival or the development of toxicity.

Furthermore, the discrepancies in ADA detection between the in-house developed acid dissociation ELISA and a conventional ELISA described in chapter 3, indicate a need for further research. Firstly, with regards to the prevalence and role of anti-ICI antibodies and secondly, with regards to the methodology applied. To determine the role and prevalence of ADAs in patients undergoing ICI-therapy, a future analysis of samples from larger cohort of patients who experienced a range of responses including disease progression could be conducted. This would enable a more accurate representation of the prevalence of anti-ICIs in patients undergoing ICI-therapy and their association with response. Additionally, due to subtle differences in structure between anti-PD-1 agents, future research could include patients treated with nivolumab monotherapy, as well as those undergoing pembrolizumab monotherapy. In our study, limited samples from nivolumab-treated patients were available for analysis. This is because, patients treated at Christchurch hospital are

preferentially treated with pembrolizumab, which is administered is every three weeks, rather than nivolumab, which is administered every two weeks. Analysis of nivolumab, as well as pembrolizumab treated patients will enable a better characterization of, not only anti-PD-1 agents as a whole, but also individual therapeutic antibodies and their respective anti-drug antibodies.

It is clear from this study, that the in-house developed ACE-ELISA possesses superior qualities to that of the commonly used bridging ELISA. The ability of the ACE-ELISA described in chapter 3, to detect ADAs that were not detected by a conventional bridging ELISA method, warrants the further development of the method. Although we are not there yet, the routine monitoring of ICIs and anti-ICIs can only be introduced into general practice, given the availability of appropriate methodology. Future studies applying this method to various patient populations and subsequently, confirming positive (and negative) samples as true positives (or negatives) should be conducted. This will provide a better representation of the accuracy of this technique, with regards to its specificity and sensitivity, and its potential for clinical use in patients treated with therapeutic mAbs.

4.3 Conclusion

In this study, no clinicopathological or immunogenic biomarkers were found to reliably predict patient response, survival or the development of toxicity. However, two key observations were made. Firstly, patients excluded from initial clinical trials of anti-PD-1 agents may be effectively treated with ICIs and secondly, commonly used bridging ELISAs for detection of drug and anti-drug parameters may inaccurately detect the production of anti-ICI antibodies. The observations from this study provide an insight into real-life metastatic melanoma patients treated with anti-PD-1 monotherapy, and importantly, constitute a step towards the optimization of immune checkpoint inhibitors in clinical practice. If the potential role of anti-drug antibodies during ICI-therapy can be established, then monitoring of ADAs may be useful in guiding individual therapy, ultimately, resulting in improved outcomes of patients treated with immune checkpoint inhibitors.

5 References

[1] Ascierto PA, Marincola FM. 2015: the year of anti-PD-1/PD-L1s against melanoma and beyond. EBioMedicine. 2015;2(2):92-3.

[2] Michot JM, Bigenwald C, Champiat S, Collins M, Carbonnel F, Postel-Vinay S, et al. Immune-related adverse events with immune checkpoint blockade: a comprehensive review. Eur J Cancer. 2016;54:139-48.

[3] Coley WB. II. Contribution to the Knowledge of Sarcoma. Ann Surg. 1891;14(3):199-220.

[4] Coley WB. The treatment of inoperable sarcoma with the 'mixed toxins of erysipelas and bacillus prodigiosus. Immediate and final results in one hundred and forty cases. Journal of the American Medical Association. 1898;XXXI(9):456-65.

[5] Sweis RF, Luke JJ. Mechanistic and pharmacologic insights on immune checkpoint inhibitors. Pharmacol Res. 2017;120:1-9.

[6] Sondak VK, Liu P-Y, Tuthill RJ, Kempf RA, Unger JM, Sosman JA, et al. Adjuvant immunotherapy of resected, intermediate-thickness, node-negative melanoma with an allogeneic tumor vaccine: overall results of a randomized trial of the Southwest Oncology Group. Journal of Clinical Oncology. 2002;20(8):2058-66.

[7] Sathyanarayanan V, Neelapu SS. Cancer immunotherapy: Strategies for personalization and combinatorial approaches. Molecular oncology. 2015;9(10):2043-53.

[8] Louis CU, Savoldo B, Dotti G, Pule M, Yvon E, Myers GD, et al. Antitumor activity and long-term fate of chimeric antigen receptor–positive T cells in patients with neuroblastoma. Blood. 2011;118(23):6050-6.

[9] Gettinger SN, Horn L, Gandhi L, Spigel DR, Antonia SJ, Rizvi NA, et al. Overall survival and long-term safety of nivolumab (anti–programmed death 1 antibody, BMS-936558, ONO-4538) in patients with previously treated advanced non–small-cell lung cancer. Journal of clinical oncology. 2015;33(18):2004-12.

[10] Hodi FS, O'day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl j Med. 2010;2010(363):711-23.

[11] Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, activity, and immune correlates of anti–PD-1 antibody in cancer. New England Journal of Medicine. 2012;366(26):2443-54.

[12] Ansell SM, Lesokhin AM, Borrello I, Halwani A, Scott EC, Gutierrez M, et al. PD-1 Blockade with Nivolumab in Relapsed or Refractory Hodgkin's Lymphoma. New England Journal of Medicine. 2015;372(4):311-9.

[13] Corsello SM, Barnabei A, Marchetti P, De Vecchis L, Salvatori R, Torino F. Endocrine Side Effects Induced by Immune Checkpoint Inhibitors. The Journal of Clinical Endocrinology & Metabolism. 2013;98(4):1361-75.

[14] June CH, Warshauer JT, Bluestone JA. Is autoimmunity the Achilles' heel of cancer immunotherapy? Nature medicine. 2017;23(5):540-7.

[15] Iwama S, De Remigis A, Callahan MK, Slovin SF, Wolchok JD, Caturegli P. Pituitary Expression of CTLA-4 Mediates Hypophysitis Secondary to Administration of CTLA-4 Blocking Antibody. Science Translational Medicine. 2014;6(230):230ra45.

[16] Minkis K, Garden BC, Wu S, Pulitzer MP, Lacouture ME. The risk of rash associated with ipilimumab in patients with cancer: A systematic review of the literature and meta-analysis. Journal of the American Academy of Dermatology. 2013;69(3):e121-e8.

[17] Naidoo J, Page D, Li B, Connell L, Schindler K, Lacouture M, et al. Toxicities of the anti-PD-1 and anti-PD-L1 immune checkpoint antibodies. Annals of Oncology. 2015;26(12):2375-91.

[18] Abdel-Rahman O, Helbling D, Schmidt J, Petrausch U, Giryes A, Mehrabi A, et al. Treatment-related Death in Cancer Patients Treated with Immune Checkpoint Inhibitors: A Systematic Review and Meta-analysis. Clinical Oncology. 2016.

[19] Weber JS, D'Angelo SP, Minor D, Hodi FS, Gutzmer R, Neyns B, et al. Nivolumab versus chemotherapy in patients with advanced melanoma who progressed after anti-CTLA-4 treatment (CheckMate 037): a randomised, controlled, open-label, phase 3 trial. The Lancet Oncology. 2015;16(4):375-84.

[20] Ribas A, Puzanov I, Dummer R, Schadendorf D, Hamid O, Robert C, et al. Pembrolizumab versus investigator-choice chemotherapy for ipilimumab-refractory melanoma (KEYNOTE-002): a randomised, controlled, phase 2 trial. The Lancet Oncology.16(8):908-18.

[21] Robert C, Schachter J, Long GV, Arance A, Grob JJ, Mortier L, et al. Pembrolizumab versus Ipilimumab in Advanced Melanoma. New England Journal of Medicine. 2015;372(26):2521-32.

[22] Burnet M. Cancer - A Biological Approach. Br Med J. 1957;1(5023):841-7.

[23] Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. Nature immunology. 2002;3(11):991-8.

[24] Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science. 2011;331(6024):1565-70.

[25] Dunn GP, Bruce AT, Sheehan KC, Shankaran V, Uppaluri R, Bui JD, et al. A critical function for type I interferons in cancer immunoediting. Nature immunology. 2005;6(7):722-9.

[26] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. cell. 2011;144(5):646-74.

[27] Vinay DS, Ryan EP, Pawelec G, Talib WH, Stagg J, Elkord E, et al. Immune evasion in cancer: Mechanistic basis and therapeutic strategies. Seminars in Cancer Biology. 2015;35, Supplement:S185-S98.

[28] Tumeh PC, Harview CL, Yearley JH, Shintaku IP, Taylor EJM, Robert L, et al. PD-1 blockade induces responses by inhibiting adaptive immune resistance. Nature. 2014;515(7528):568-71.

[29] Spranger S, Spaapen RM, Zha Y, Williams J, Meng Y, Ha TT, et al. Up-Regulation of PD-L1, IDO, and Tregs in the Melanoma Tumor Microenvironment Is Driven by CD8+ T Cells. Science Translational Medicine. 2013;5(200):116-200.

[30] Fife BT, Bluestone JA. Control of peripheral T-cell tolerance and autoimmunity via the CTLA-4 and PD-1 pathways. Immunological Reviews. 2008;224(1):166-82.

[31] Bretscher P, Cohn M. A theory of self-nonself discrimination. Science. 1970;169(3950):1042-9.

[32] Lafferty KJ, Cunningham A. A new analysis of allogeneic interactions. Australian Journal of Experimental Biology & Medical Science. 1975;53(1).

[33] Chen L, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. Nature Reviews Immunology. 2013;13(4):227-42.

[34] Walunas TL, Lenschow DJ, Bakker CY, Linsley PS, Freeman GJ, Green JM, et al. CTLA-4 can function as a negative regulator of T cell activation. Immunity. 1994;1(5):405-13.

[35] Wang C, Thudium KB, Han M, Wang X-T, Huang H, Feingersh D, et al. In vitro characterization of the anti-PD-1 antibody nivolumab, BMS-936558, and in vivo toxicology in non-human primates. Cancer immunology research. 2014;2(9):846-56.

[36] Karandikar NJ, Vanderlugt CL, Walunas TL, Miller SD, Bluestone JA. CTLA-4: a negative regulator of autoimmune disease. Journal of Experimental Medicine. 1996;184(2):783-8.

[37] Wang J, Yoshida T, Nakaki F, Hiai H, Okazaki T, Honjo T. Establishment of NOD-Pdcd1-/-mice as an efficient animal model of type I diabetes. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(33):11823-8.

[38] Waterhouse P, Penninger JM, Timms E, Wakeham A. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. Science. 1995;270(5238):985.

[39] Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity. 1995;3(5):541-7.

[40] Nishimura H, Nose M, Hiai H, Minato N, Honjo T. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. Immunity. 1999;11(2):141-51.

[41] Nishimura H, Okazaki T, Tanaka Y, Nakatani K, Hara M, Matsumori A, et al. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. Science. 2001;291(5502):319-22.

[42] Salama AD, Chitnis T, Imitola J, Ansari MJI, Akiba H, Tushima F, et al. Critical role of the programmed death-1 (PD-1) pathway in regulation of experimental autoimmune encephalomyelitis. Journal of Experimental Medicine. 2003;198(1):71-8.

[43] Nishimura H, Minato N, Nakano T, Honjo T. Immunological studies on PD-1 deficient mice: implication of PD-1 as a negative regulator for B cell responses. International immunology. 1998;10(10):1563-72.

[44] Lin SC, Yen JH, Tsai JJ, Tsai WC, Ou TT, Liu HW, et al. Association of a programmed death 1 gene polymorphism with the development of rheumatoid arthritis, but not systemic lupus erythematosus. Arthritis & Rheumatism. 2004;50(3):770-5.

[45] Djilali-Saiah I, Schmitz J, Harfouch-Hammoud E, Mougenot J, Bach J, Caillat-Zucman S. CTLA-4 gene polymorphism is associated with predisposition to coeliac disease. Gut. 1998;43(2):187-9.

[46] Parry RV, Chemnitz JM, Frauwirth KA, Lanfranco AR, Braunstein I, Kobayashi SV, et al. CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. Molecular and cellular biology. 2005;25(21):9543-53.

[47] Buchbinder EI, Desai A. CTLA-4 and PD-1 pathways: similarities, differences, and implications of their inhibition. American journal of clinical oncology. 2016;39(1):98.

[48] Collins AV, Brodie DW, Gilbert RJ, Iaboni A, Manso-Sancho R, Walse B, et al. The interaction properties of costimulatory molecules revisited. Immunity. 2002;17(2):201-10.

[49] Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. Journal of Experimental Medicine. 2000;192(7):1027-34.

[50] Latchman Y, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I, et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. Nature immunology. 2001;2(3):261-8.

[51] Keir ME, Francisco LM, Sharpe AH. PD-1 and its ligands in T-cell immunity. Current opinion in immunology. 2007;19(3):309-14.

[52] Thompson RH, Gillett MD, Cheville JC, Lohse CM, Dong H, Webster WS, et al. Costimulatory B7-H1 in renal cell carcinoma patients: Indicator of tumor aggressiveness and potential therapeutic target. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(49):17174-9.

[53] Karim R, Jordanova ES, Piersma SJ, Kenter GG, Chen L, Boer JM, et al. Tumor-expressed B7-H1 and B7-DC in relation to PD-1+ T-cell infiltration and survival of patients with cervical carcinoma. Clinical Cancer Research. 2009;15(20):6341-7.

[54] Nomi T, Sho M, Akahori T, Hamada K, Kubo A, Kanehiro H, et al. Clinical significance and therapeutic potential of the programmed death-1 ligand/programmed death-1 pathway in human pancreatic cancer. Clinical cancer research. 2007;13(7):2151-7.

[55] Obeid JM, Erdag G, Smolkin ME, Deacon DH, Patterson JW, Chen L, et al. PD-L1, PD-L2 and PD-1 expression in metastatic melanoma: Correlation with tumor-infiltrating immune cells and clinical outcome. OncoImmunology. 2016;5(11):e1235107.

[56] Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. Nature medicine. 2002;8(8):793-800.

[57] Iwai Y, Ishida M, Tanaka Y, Okazaki T, Honjo T, Minato N. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. Proceedings of the National Academy of Sciences. 2002;99(19):12293-7.

[58] Chen DS, Mellman I. Oncology meets immunology: the cancer-immunity cycle. Immunity. 2013;39(1):1-10.

[59] Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. Science. 1996;271(5256):1734.

[60] Hirano F, Kaneko K, Tamura H, Dong H, Wang S, Ichikawa M, et al. Blockade of B7-H1 and PD-1 by monoclonal antibodies potentiates cancer therapeutic immunity. Cancer research. 2005;65(3):1089-96.

[61] Harper K, Balzano C, Rouvier E, Mattéi M-G, Luciani M, Golstein P. CTLA-4 and CD28 activated lymphocyte molecules are closely related in both mouse and human as to sequence, message expression, gene structure, and chromosomal location. The Journal of Immunology. 1991;147(3):1037-44.

[62] Ishida Y, Agata Y, Shibahara K, Honjo T. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. The EMBO Journal. 1992;11(11):3887-95.

[63] Stamper CC, Zhang Y, Tobin JF, Erbe DV, Ikemizu S, Davis SJ, et al. Crystal structure of the B7-1/CTLA-4 complex that inhibits human immune responses. Nature. 2001;410(6828):608-11.

[64] Avelumab (BAVENCIO). U.S. Food & Drug Administration (FDA). 2017; https://www.fda.gov/drugs/informationondrugs/approveddrugs/ucm547965.htm

[65] Durvalumab (Imfinzi). U.S. Food & Drug Administration (FDA). 2017; https://www.fda.gov/drugs/informationondrugs/approveddrugs/ucm555930.htm

[66] El-Khoueiry AB, Melero I, Crocenzi TS, Welling TH, Yau TC, Yeo W, et al. Phase I/II safety and antitumor activity of nivolumab in patients with advanced hepatocellular carcinoma (HCC): CA209-040. American Society of Clinical Oncology; 2015.

[67] Daver N, Basu S, Garcia-Manero G, Cortes JE, Ravandi F, Jabbour EJ, et al. Phase IB/II Study of Nivolumab in Combination with Azacytidine (AZA) in Patients (pts) with Relapsed Acute Myeloid Leukemia (AML). Am Soc Hematology; 2016.

[68] Ceresoli GL, Mantovani A. Immune checkpoint inhibitors in malignant pleural mesothelioma. The Lancet Oncology. 2017;18(5):559-61.

[69] Leidner R, Kang H, Haddad R, Segal NH, Wirth LJ, Ferris RL, et al., editors. Preliminary efficacy from a phase 1/2 study of the natural killer cell–targeted antibody, lirilumab in combination with nivolumab in squamous cell carcinoma of the head and neck. SITC Annual Meeting; 2016.

[70] Sanborn RE, Sharfman WH, Segal NH, Hodi FS, Wolchok JD, Urba WJ, et al. A phase I dose-escalation and cohort expansion study of lirilumab (anti-KIR; BMS-986015) administered in combination with nivolumab (anti-PD-1; BMS-936558; ONO-4538) in patients (Pts) with advanced refractory solid tumors. J Clin Oncol. 2013;31:suppl; abstr TPS3110.

[71] Ipilimumab (Yervoy®) [prescribing information]. Princeton, NJ: Bristol-Myers Squibb Company; 2013.

[72] Kwon ED, Drake CG, Scher HI, Fizazi K, Bossi A, Van den Eertwegh AJ, et al. Ipilimumab versus placebo after radiotherapy in patients with metastatic castration-resistant prostate cancer that had progressed after docetaxel chemotherapy (CA184-043): a multicentre, randomised, double-blind, phase 3 trial. The lancet oncology. 2014;15(7):700-12.

[73] Reck M, Luft A, Szczesna A, Havel L, Kim S-W, Akerley W, et al. Phase III randomized trial of ipilimumab plus etoposide and platinum versus placebo plus etoposide and platinum in extensive-stage small-cell lung cancer. Journal of Clinical Oncology. 2016:JCO676601.

[74] Royal RE, Levy C, Turner K, Mathur A, Hughes M, Kammula US, et al. Phase 2 trial of single agent Ipilimumab (anti-CTLA-4) for locally advanced or metastatic pancreatic adenocarcinoma. Journal of immunotherapy. 2010;33(8):828-33

[75] Nivolumab (Opdivo) Prescribing information. U.S. Food & Drug Administration (FDA). 2016; https://www.accessdata.fda.gov/drugsatfda_docs/label/2016/125554s019lbl.pdf

[76] Pembrolizumab (Keytruda) Prescribing information. U.S. Food & Drug Administration (FDA). 2017; https://www.accessdata.fda.gov/drugsatfda_docs/label/2017/125514s016lbl.pdf

[77] Clinicaltrials.gov. Cited 31 May 2017; https://clinicaltrials.gov/ct2/results?term=immune+checkpoint+inhibitor+combination&Search=Search

[78] Larkin J, Chiarion-Sileni V, Gonzalez R, Grob JJ, Cowey CL, Lao CD, et al. Combined nivolumab and ipilimumab or monotherapy in untreated melanoma. N Engl J Med. 2015;2015(373):23-34.

[79] Common terminology criteria for adverse events (CTCAE) Version 4.0. 2009; https://evs.nci.nih.gov/ftp1/CTCAE/CTCAE_4.03_2010-06-14_QuickReference_5x7.pdf

[80] Chen T, Razak A, Bedard P, Siu L, Hansen A. A systematic review of immune-related adverse event reporting in clinical trials of immune checkpoint inhibitors. Annals of Oncology. 2015;26(9):1824-9.

[81] Maughan BL, Bailey E, Gill DM, Agarwal N. Incidence of Immune-Related Adverse Events with Program Death Receptor-1-and Program Death Receptor-1 Ligand-Directed Therapies in Genitourinary Cancers. Frontiers in Oncology. 2017;7.

[82] Caspi RR. Immunotherapy of autoimmunity and cancer: the penalty for success. Nature reviews immunology. 2008;8(12):970-6.

[83] June CH, Warshauer JT, Bluestone JA. Is autoimmunity the Achilles' heel of cancer immunotherapy? Nature medicine. 2017;23(5):540-7.

[84] Ngo S, Steyn F, McCombe P. Gender differences in autoimmune disease. Frontiers in neuroendocrinology. 2014;35(3):347-69.

[85] Goronzy JJ, Weyand CM. Immune aging and autoimmunity. Cellular and Molecular Life Sciences. 2012;69(10):1615-23.

[86] Bertrand A, Kostine M, Barnetche T, Truchetet M-E, Schaeverbeke T. Immune related adverse events associated with anti-CTLA-4 antibodies: systematic review and meta-analysis. BMC medicine. 2015;13(1):211.

[87] Sibaud V, Meyer N, Lamant L, Vigarios E, Mazieres J, Delord JP. Dermatologic complications of anti-PD-1/PD-L1 immune checkpoint antibodies. Current opinion in oncology. 2016;28(4):254-63.

[88] Ibrahim R, Berman D, DePril V, Humphrey R, Chen T, Messina M, et al. Ipilimumab safety profile: Summary of findings from completed trials in advanced melanoma. Journal of Clinical Oncology. 2011;29(15_suppl):8583-.

[89] Boutros C, Tarhini A, Routier E, Lambotte O, Ladurie FL, Carbonnel F, et al. Safety profiles of anti-CTLA-4 and anti-PD-1 antibodies alone and in combination. Nature Reviews Clinical Oncology. 2016.

[90] Lo JA, Fisher DE, Flaherty KT. Prognostic significance of cutaneous adverse events associated with pembrolizumab therapy. JAMA oncology. 2015;1(9):1340-1.

[91] Ram M, Shoenfeld Y. Harnessing Autoimmunity (Vitiligo) to Treat Melanoma. Annals of the New York Academy of Sciences. 2007;1110(1):410-25.

[92] Houghton A, Eisinger M, Albino AP, Cairncross J, Old L. Surface antigens of melanocytes and melanomas. Markers of melanocyte differentiation and melanoma subsets. Journal of Experimental Medicine. 1982;156(6):1755-66.

[93] Uenami T, Hosono Y, Ishijima M, Kanazu M, Akazawa Y, Yano Y, et al. Vitiligo in a patient with lung adenocarcinoma treated with nivolumab: A case report. Lung Cancer. 2017;109:42-4.

[94] Yin ES, Totonchy MB, Leventhal JS. Nivolumab-associated vitiligo-like depigmentation in a patient with acute myeloid leukemia: A novel finding. JAAD Case Reports. 2017;3(2):90.

[95] Spain L, Diem S, Larkin J. Management of toxicities of immune checkpoint inhibitors. Cancer treatment reviews. 2016;44:51-60.

[96] Ribas A, Puzanov I, Dummer R, Schadendorf D, Hamid O, Robert C, et al. Pembrolizumab versus investigator-choice chemotherapy for ipilimumab-refractory melanoma (KEYNOTE-002): a randomised, controlled, phase 2 trial. The lancet oncology. 2015;16(8):908-18.

[97] Weber JS, Kudchadkar RR, Yu B, Gallenstein D, Horak CE, Inzunza HD, et al. Safety, efficacy, and biomarkers of nivolumab with vaccine in ipilimumab-refractory or-naive melanoma. Journal of clinical oncology. 2013;31(34):4311-8.

[98] Kong Y-cM, Flynn JC. Opportunistic autoimmune disorders potentiated by immune-checkpoint inhibitors anti-CTLA-4 and anti-PD-1. Frontiers in immunology. 2014;5:206.

[99] Carbognin L, Pilotto S, Milella M, Vaccaro V, Brunelli M, Caliò A, et al. Differential activity of nivolumab, pembrolizumab and MPDL3280A according to the tumor expression of programmed

death-ligand-1 (PD-L1): sensitivity analysis of trials in melanoma, lung and genitourinary cancers. PloS one. 2015;10(6):e0130142.

[100] Attia P, Phan GQ, Maker AV, Robinson MR, Quezado MM, Yang JC, et al. Autoimmunity correlates with tumor regression in patients with metastatic melanoma treated with anti–cytotoxic T-lymphocyte antigen-4. Journal of Clinical Oncology. 2005;23(25):6043-53.

[101] Teulings H-E, Limpens J, Jansen SN, Zwinderman AH, Reitsma JB, Spuls PI, et al. Vitiligo-like depigmentation in patients with stage III-IV melanoma receiving immunotherapy and its association with survival: a systematic review and meta-analysis. Journal of Clinical Oncology. 2015;33(7):773-81.

[102] Nordlund JJ, Kirkwood JM, Forget BM, Milton G, Albert DM, Lerner AB. Vitiligo in patients with metastatic melanoma: a good prognostic sign. Journal of the American Academy of Dermatology. 1983;9(5):689-96.

[103] Hua C, Boussemart L, Mateus C, Routier E, Boutros C, Cazenave H, et al. Association of vitiligo with tumor response in patients with metastatic melanoma treated with pembrolizumab. JAMA dermatology. 2016;152(1):45-51.

[104] Freeman-Keller M, Kim Y, Cronin H, Richards A, Gibney G, Weber JS. Nivolumab in resected and unresectable metastatic melanoma: characteristics of immune-related adverse events and association with outcomes. Clinical Cancer Research. 2016;22(4):886-94.

[105] Sanlorenzo M, Vujic I, Daud A, Algazi A, Gubens M, Luna SA, et al. Pembrolizumab cutaneous adverse events and their association with disease progression. JAMA dermatology. 2015;151(11):1206-12.

[106] Phan GQ, Attia P, Steinberg SM, White DE, Rosenberg SA. Factors associated with response to high-dose interleukin-2 in patients with metastatic melanoma. Journal of Clinical Oncology. 2001;19(15):3477-82.

[107] Downey SG, Klapper JA, Smith FO, Yang JC, Sherry RM, Royal RE, et al. Prognostic factors related to clinical response in patients with metastatic melanoma treated by CTL-associated antigen-4 blockade. Clinical Cancer Research. 2007;13(22):6681-8.

[108] Khoja L, Atenafu EG, Templeton A, Qye Y, Chappell MA, Saibil S, et al. The full blood count as a biomarker of outcome and toxicity in ipilimumab-treated cutaneous metastatic melanoma. Cancer Medicine. 2016;5(10):2792-9.

[109] Horvat TZ, Adel NG, Dang T-O, Momtaz P, Postow MA, Callahan MK, et al. Immune-related adverse events, need for systemic immunosuppression, and effects on survival and time to treatment failure in patients with melanoma treated with ipilimumab at Memorial Sloan Kettering Cancer Center. Journal of Clinical Oncology. 2015;33(28):3193-8.

[110] Gyorki DE, Callahan M, Wolchok JD, Ariyan CE. The delicate balance of melanoma immunotherapy. Clinical & translational immunology. 2013;2(8):e5.

[111] Yervoy Risk Evaluation and Mitigation Strategy (REMS). 2011; https://www.accessdata.fda.gov/drugsatfda_docs/nda/2011/125377orig1s000rems.pdf

[112] Kefford R, Ribas A, Hamid O, Robert C, Daud A, Wolchok JD, et al. Clinical efficacy and correlation with tumor PD-L1 expression in patients (pts) with melanoma (MEL) treated with the anti-PD-1 monoclonal antibody MK-3475. American Society of Clinical Oncology; 2014.
[113] Taube JM, Klein A, Brahmer JR, Xu H, Pan X, Kim JH, et al. Association of PD-1, PD-1 ligands, and other features of the tumor immune microenvironment with response to anti–PD-1 therapy. Clinical cancer research. 2014;20(19):5064-74.

[114] Borghaei H, Paz-Ares L, Horn L, Spigel DR, Steins M, Ready NE, et al. Nivolumab versus docetaxel in advanced nonsquamous non–small-cell lung cancer. New England Journal of Medicine. 2015;373(17):1627-39.

[115] Gandini S, Massi D, Mandalà M. PD-L1 expression in cancer patients receiving anti PD-1/PD-L1 antibodies: A systematic review and meta-analysis. Critical reviews in oncology/hematology. 2016;100:88-98.

[116] Kluger HM, Zito CR, Turcu G, Baine M, Zhang H, Adeniran A, et al. PD-L1 Studies Across Tumor Types, its Differential Expression and Predictive Value in Patients Treated with Immune Checkpoint Inhibitors. Clinical Cancer Research. 2017:clincanres. 3146.2016.

[117] Wang X, Teng F, Kong L, Yu J. PD-L1 expression in human cancers and its association with clinical outcomes. OncoTargets and therapy. 2016;9:5023.

[118] McLaughlin J, Han G, Schalper KA, Carvajal-Hausdorf D, Pelekanou V, Rehman J, et al. Quantitative assessment of the heterogeneity of PD-L1 expression in non–small-cell lung cancer. JAMA oncology. 2016;2(1):46-54.

[119] Snyder A, Makarov V, Merghoub T, Yuan J, Zaretsky JM, Desrichard A, et al. Genetic basis for clinical response to CTLA-4 blockade in melanoma. New England Journal of Medicine. 2014;371(23):2189-99.

[120] Sivan A, Corrales L, Hubert N, Williams JB, Aquino-Michaels K, Earley ZM, et al. Commensal Bifidobacterium promotes antitumor immunity and facilitates anti–PD-L1 efficacy. Science. 2015;350(6264):1084-9.

[121] Rizvi NA, Hellmann MD, Snyder A, Kvistborg P, Makarov V, Havel JJ, et al. Mutational landscape determines sensitivity to PD-1 blockade in non–small cell lung cancer. Science. 2015;348(6230):124-8.

[122] Zaretsky JM, Garcia-Diaz A, Shin DS, Escuin-Ordinas H, Hugo W, Hu-Lieskovan S, et al. Mutations associated with acquired resistance to PD-1 blockade in melanoma. New England Journal of Medicine. 2016;375(9):819-29.

[123] ELCC 2017 Press Release: White Blood Cell Count Predicts Response to Lung Cancer Immunotherapy 2017; http://esmo.org/Conferences/ELCC-2017-Lung-Cancer/News-Press-Releases/White-Blood-Cell-Count-Predicts-Response-to-Lung-Cancer-Immunotherapy

[124] Gibney GT, Weiner LM, Atkins MB. Predictive biomarkers for checkpoint inhibitor-based immunotherapy. The Lancet Oncology. 2016;17(12):e542-e51.

[125] Shahabi V, Berman D, Chasalow SD, Wang L, Tsuchihashi Z, Hu B, et al. Gene expression profiling of whole blood in ipilimumab-treated patients for identification of potential biomarkers of immune-related gastrointestinal adverse events. Journal of translational medicine. 2013;11(1):75.

[126] Berman D, Parker SM, Siegel J, Chasalow SD, Weber J, Galbraith S, et al. Blockade of cytotoxic T-lymphocyte antigen-4 by ipilimumab results in dysregulation of gastrointestinal immunity in patients with advanced melanoma. Cancer Immunity Archive. 2010;10(1):11.

[127] Callahan M, Yang A, Tandon S, Xu Y, Subudhi S, Roman R, et al. Evaluation of serum IL-17 levels during ipilimumab therapy: Correlation with colitis. Journal of Clinical Oncology. 2011;29(15_suppl):2505-.

[128] Tarhini AA, Zahoor H, Lin Y, Malhotra U, Sander C, Butterfield LH, et al. Baseline circulating IL-17 predicts toxicity while TGF-β1 and IL-10 are prognostic of relapse in ipilimumab neoadjuvant therapy of melanoma. Journal for immunotherapy of cancer. 2015;3(1):39.

[129] Schindler K, Harmankaya K, Kuk D, Mangana J, Michielin O, Hoeller C, et al. Correlation of absolute and relative eosinophil counts with immune-related adverse events in melanoma patients treated with ipilimumab. American Society of Clinical Oncology; 2014.

[130] Thebeau M, Rubin K, Hofmann M, Grimm J, Weinstein A, Choi JN. Management of skin adverse events associated with immune checkpoint inhibitors in patients with melanoma: A nursing perspective. Journal of the American Association of Nurse Practitioners. 2017;29(5):294-303.

[131] Kumar V, Chaudhary N, Garg M, Floudas CS, Soni P, Chandra AB. Current diagnosis and management of immune related adverse events (irAEs) induced by immune checkpoint inhibitor therapy. Frontiers in pharmacology. 2017;8.

[132] Hamanishi J, Mandai M, Matsumura N, Abiko K, Baba T, Konishi I. PD-1/PD-L1 blockade in cancer treatment: perspectives and issues. International journal of clinical oncology. 2016;21(3):462-73.

[133] Achkar T, Tarhini AA. The use of immunotherapy in the treatment of melanoma. Journal of Hematology & Oncology. 2017;10(1):88.

[134] Weber JS, Kähler KC, Hauschild A. Management of immune-related adverse events and kinetics of response with ipilimumab. Journal of Clinical Oncology. 2012;30(21):2691-7.

[135] Nishino M. Immune-related response evaluations during immune-checkpoint inhibitor therapy: establishing a “common language” for the new arena of cancer treatment. Journal for ImmunoTherapy of Cancer. 2016 Jun 21;4(1):30.

[136] Chames P, Van Regenmortel M, Weiss E, Baty D. Therapeutic antibodies: successes, limitations and hopes for the future. Br J Pharmacol. 2009;157(2):220-33.

[137] Ryman JT, Meibohm B. Pharmacokinetics of Monoclonal Antibodies. CPT Pharmacometrics Syst Pharmacol. 2017.

[138] Immunoglobulin Structure and Classes; https://www.thermofisher.com/nz/en/home/life-science/antibodies/antibodies-learning-center/antibodies-resource-library/antibody-methods/immunoglobulin-structure-classes.html

[140] Vultaggio A, Matucci A, Nencini F, Pratesi S, Parronchi P, Rossi O et al. Anti-infliximab IgE and non-IgE antibodies and induction of infusion-related severe anaphylactic reactions. Allergy. 2010;65(5):657-61.

[141] Bourdage JS, Cook CA, Farrington DL, Chain JS, Konrad RJ. An Affinity Capture Elution (ACE) assay for detection of anti-drug antibody to monoclonal antibody therapeutics in the presence of high levels of drug. J Immunol Methods. 2007;327(1):10-7.

[142] Baert F, Noman M, Vermeire S, Van Assche G, D'Haens G, Carbonez A, Rutgeerts P. Influence of immunogenicity on the long-term efficacy of infliximab in Crohn's disease. New England Journal of Medicine. 2003;348(7):601-8.

[143] Wang SL, Ohrmund L, Hauenstein S, Salbato J, Reddy R, Monk P, Lockton S, Ling N et al. Development and validation of a homogeneous mobility shift assay for the measurement of infliximab and antibodies-to-infliximab levels in patient serum. J Immunol Methods. 2012;382(1):177-88.

[144] Mok CC, Van Der Kleij D, Wolbink GJ. Drug levels, anti-drug antibodies, and clinical efficacy of the anti-TNFα biologics in rheumatic diseases. Clinical rheumatology. 2013;32(10):1429-35.

[145] West RL, Zelinkova Z, Wolbink GJ, Kuipers EJ, Stokkers PC, Van Der Woude CJ. Immunogenicity negatively influences the outcome of adalimumab treatment in Crohn's disease. Aliment Pharmacol Ther. 2008;28(9):1122-6.

[146] Ogric M, Tercelj M, Praprotnik S, Tomsic M, Bozic B, Sodin-Semri S, Cucnik S. Detection of adalimumab and anti-adalimumab antibodies in patients with rheumatoid arthritis: a comprehensive overview of methodology pitfalls and benefits. Immunol Res. 2017;65(1):172-185.

[147] Puszkiel A, Noé G, Boudou-Rouquette P, Le-Cossec C, Arrondeau J, Giraud JS, Thomas-Schoemann A et al. Development and validation of an ELISA method for the quantification of nivolumab in plasma from non-small-cell lung cancer patients. J Pharmaceut Biomed. 2017;139:30-6.

[148] European Medicines Agency (EMA) Assessment Report, Keytruda http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Public_assessment_report/human/003820/WC500190992.pdf

[149] The Cost of Skin Cancer to New Zealand, A report to The Cancer Society of New Zealand (2009). https://wellington.cancernz.org.nz/assets/Sunsmart/Information-sheets/CostsofSkinCancer-NZ-22October2009.pdf

[150] PHARMAC: Proposal to list nivolumab (Opdivo) for advanced melanoma (2016) https://www.pharmac.govt.nz/news/consultation-2016-05-04-nivolumab/

[151] Yarur AJ, Abreu MT, Deshpande AR, Kerman DH, Sussman DA. Therapeutic drug monitoring in patients with inflammatory bowel disease. WJG. 2014;20(13):3475.

[152] Bender N, Heilig C, Droll B, Wohlgemuth J, Armbruster F, Heilig B. Immunogenicity, efficacy and adverse events of adalimumab in RA patients. Rheumatol Int. 2007;27(3):269-274.

[153] Kang JS, Lee MH. Overview of therapeutic drug monitoring. Korean J Intern Med. 2009;24(1):1.

[154] Yearley JH, Gibson C, Yu N, Moon C, Murphy E, Juco J, Lunceford J, Cheng J et al. PD-L2 Expression in Human Tumors: Relevance to Anti-PD-1 Therapy in Cancer. Clin Can Res. 2017;23(12):3158-67.

[155] Hodi FS, Hwu WJ, Kefford R, Weber JS, Daud A, Hamid O, Patnaik A, Ribas A et al. Evaluation of immune-related response criteria and RECIST v1. 1 in patients with advanced melanoma treated with pembrolizumab. Journal of Clinical Oncology. 2016;34(13):1510-7.

[156] Wolchok JD, Hoos A, O'Day S, Weber JS, Hamid O, Lebbé C, Maio M, Binder M et al. Guidelines for the evaluation of immune therapy activity in solid tumors: immune-related response criteria. Clin Can Res. 2009;15(23):7412-20.

[157] Seymour L, Bogaerts J, Perrone A, Ford R, Schwartz LH, Mandrekar S, Lin NU, Litière S et al. iRECIST: guidelines for response criteria for use in trials testing immunotherapeutics. The Lancet Oncology. 2017;18(3):143-52.

[158] Murli K, Nadler S. Immunogenicity to biotherapeutics – the role of anti-drug immune complexes. Front Immunol. 2016;7.

[159] Ministry of Health NZ: Annual Update of key results 2015/16 – New Zealand Healthy Survey http://www.health.govt.nz/publication/annual-update-key-results-2015-16-new-zealand-health-survey

[160] Abdel-Wahab N, Shah M, Suarez-Almazor M. Use of Immune Checkpoint Inhibitors in the treatment of patients with cancer and preexisting autoimmune diseases: a systematic review of case reports. Arth Rhematol 2016;68.

[161] PHARMAC: Decision regarding funding of pembrolizumab (Keytruda), nivolumab (Opdivo), posaconazole (Noxafil) and raltegravir (Isentress) (2016) https://www.pharmac.govt.nz/news/notification-2016-08-02-pembrolizumab-nivolumab/

[162] Kittai AS, Oldham H, Cetnar J, Taylor M. Immune Checkpoint Inhibitors in Organ Transplant Patients. J Immunotherapy. 2017;40(7):277-81.

[163] Lerner A, Jeremias P, Matthias T. The world incidence and prevalence of autoimmune diseases is increasing. International Journal of Celiac Disease. 2015;3(4):151-5.

[164] Beck KM, Dong J, Geskin LJ, Beltrani VP, Phelps RG, Carvajal RD, Schwartz G, Saenger YM, Gartrell RD. Disease stabilization with pembrolizumab for metastatic acral melanoma in the setting of autoimmune bullous pemphigoid. Journal for immunotherapy of cancer. 2016;4(1):20.

[165] Ministry of Health NZ: Selected Cancers 2013, 2014, 2015. http://www.health.govt.nz/publication/selected-cancers-2013-2014-2015

[166] The National Cancer Intelligence Network (NCIN): Mortality, Incidence and gender – malignant melanoma (2012). http://www.ncin.org.uk/publications/data_briefings/mortality_incidence_and_gender_malignant_melanoma

[167] Elias R, Morales J, Rehman Y, Khurshid H. Immune checkpoint inhibitors in older adults. Current oncology reports. 2016;18(8):1-7.

[168] Pennock GK, Chow LQ. The evolving role of immune checkpoint inhibitors in cancer treatment. The oncologist. 2015;20(7):812-22.

[169] Haanen JB, Carbonnel F, Robert C, Kerr KM, Peters S, Larkin J, Jordan K. Management of toxicities from immunotherapy: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Annals of Oncology. 2017 ;28(4):119-42.

[170] Nishino M, Giobbie-Hurder A, Manos MP, Bailey N, Buchbinder EI, Ott PA, Ramaiya NH, Hodi FS. Immune-Related Tumor Response Dynamics in Melanoma Patients Treated with Pembrolizumab: Identifying Markers for Clinical Outcome and Treatment Decisions. Clin Can Res. 2017 Jun 7.

[171] Lugowska I, Cybulska-Stopa B, Jagodzinska-Mucha P, Teterycz P, Koseła-Paterczyk H, Kozak K, Szamotulska K, Roman K et al. Baseline neutrophil-to-lymphocyte ratio and its values monitored over time is associated with outcome of metastatic melanoma patients treated with immunotherapy. Annals of Oncology. 2017;28(5).

[172] Failing JJ, Yan Y, Porrata LF, Markovic SN. Lymphocyte-to-monocyte ratio is associated with survival in pembrolizumab-treated metastatic melanoma patients. Melanoma Research. 2017 Oct 9.

[173] Bendtzen K. Personalized medicine: theranostics (therapeutics diagnostics) essential for rational use of tumor necrosis factor-alpha antagonists. Discovery medicine. 2013;15(83):201-11.

[174] Steenholdt C, Bendtzen K, Brynskov J, et al. Clinical implications of measuring drug and anti-drug antibodies by different assays when optimizing infliximab treatment failure in Crohn's disease: post hoc analysis of a randomized controlled trial. Am J Gastroenterol. 2014;109:1055–1064.

[175] Ahamadi M, Freshwater T, Prohn M, Li CH, De Alwis DP, De Greef R, Elassaiss-Schaap J, Kondic A et al. Model-Based Characterization of the Pharmacokinetics of Pembrolizumab: A Humanized Anti–PD-1 Monoclonal Antibody in Advanced Solid Tumors. CPT: pharmacometrics & systems pharmacology. 2017;6(1):49-57.

[176] Chauhan AK, Chen C, Moore TL, DiPaolo RJ. Induced expression of FcRIIIa (CD16a) on CD4 T cells triggers generation of IFN-γ high subset. J Biol Chem (2015) 290(8):5127–40.

[177] Sanmamed MF, Perez-Gracia JL, Schalper KA, Fusco JP, Gonzalez A, Rodriguez-Ruiz ME, Oñate C, Perez G, Alfaro C, Martín-Algarra S, Andueza MP. Changes in serum interleukin-8 (IL-8) levels reflect and predict response to anti-PD-1 treatment in melanoma and non-small cell lung cancer patients. Annals of Oncology. 2017 Jun 8.

[178] Hock BD, Stamp LK, Hayman MW, Keating PE, Helms ET, Barclay ML. Development of an ELISA-based competitive binding assay for the analysis of drug concentration and antidrug antibody levels in patients receiving adalimumab or infliximab. Therapeutic drug monitoring. 2016;38(1):32-41.

[179] Bonigen J, Raynaud-Donzel C, Hureaux J, Kramkimel N, Blom A, Jeudy G, Breton AL, Hubiche T et al. Anti-PD1-induced psoriasis: a study of 21 patients. Journal of the European Academy of Dermatology and Venereology. 2017;31(5).

[180] Tanaka R, Okiyama N, Okune M, Ishitsuka Y, Watanabe R, Furuta J, Ohtsuka M, Otsuka A et al. Serum level of interleukin-6 is increased in nivolumab-associated psoriasiform dermatitis and tumor necrosis factor-α is a biomarker of nivolumab recativity. Journal of dermatological science. 2017;86(1):71-3.

[181] Weber JS, Antonia SJ, Topalian SL, Schadendorf D, Larkin JM, Sznol M, Liu HY, Waxman I, Robert C. Safety profile of nivolumab (NIVO) in patients (pts) with advanced melanoma (MEL): A pooled analysis. 2015;9018-9018

[182] Gangadhar TC, Mehnert J, Patnaik A, Hamid O, Carlino MS, Hodi FS, Blank CU, Ribas A et al. Population pharmacokinetic (popPK) model of pembrolizumab (pembro; MK-3475) in patients (pts) treated in KEYNOTE-001 and KEYNOTE-002. 2015;3058-3058